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Social Effects of Inbreeding Associated With Parental Care

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Contents

Lay Summary

When two parents are related to one another, the inbred offspring suffer negative effects known as inbreeding depression, which can have long-term costs throughout life. Inbreeding depression is generally expected when individuals themselves are inbred, but outbred individuals may also suffer inbreeding depression when they depend on other inbred individuals. The burying beetle breeds on small bird and mammal carcasses and both parents provide elaborate parental care that includes regurgitating the carcass as food to begging larvae, improving their offspring's survival. I found that inbred female parents produce offspring who do not survive as well when parents are providing care. Therefore, the negative effects of inbreeding in parents affected the next generation of offspring, who are not inbred themselves, known as an intergenerational effects. I investigated whether such intergenerational effects of inbreeding resulted from parents providing less care. I found that inbred parents provided as much care as outbred parents, but found that inbreeding in one parent did affect how much care their partner provided. In addition, I investigated the effects of inbreeding on offspring begging, a form of communication between parents and offspring, when either female parents or their offspring were inbred. I found that whilst inbred offspring begged to parents less than outbred offspring, parents provided these inbred offspring more care, suggesting that parents do not solely rely on how much offspring beg to determine how much care to provide. To further investigate the intergenerational effects of inbreeding I tested the immune defences of parents. Parents in the burying beetle apply secretions to the carcass throughout the offspring's development, which protects the carcass from decomposition and the offspring from pathogens. I found that the secretions of inbred male parents were worse at killing bacteria than outbred male parents, though I found no differences in the bacteria killing effects of female parents. Finally, to test whether the strong inbreeding effects in this species determined whether related individuals avoided inbreeding, I presented females with a related or unrelated male, and found no evidence that females avoided inbreeding. In conclusion, I show that outbred individuals may also suffer negative effects of inbreeding when they interact with those that are inbred, and to fully account for the consequences of inbreeding, the social effects of inbreeding within a family need to be considered.

Abstract

Inbreeding is associated with reduced fitness, a phenomenon known as inbreeding depression. I investigated direct and indirect effects of inbreeding on social traits associated with parental care in the burying beetle, *Nicrophorus vespilloides*. This species breeds on small vertebrate carcasses and the parents provide care by maintaining the carcass and regurgitating food to begging larvae. I quantified the survival of outbred offspring produced by inbred and outbred parents. I found that inbred offspring had reduced survival compared to outbred offspring, and that outbred offspring produced by inbred parents survived less well. Such intergenerational effects of inbreeding suggests that inbreeding may affect the amount of parental care provided to offspring. I tested this by investigating the amount of care inbred and outbred male and female parents provided to outbred offspring. I found no reductions in the amount of care provided by inbred parents but found that parents provided more care when their partner was inbred. In addition, I investigated effects of inbreeding on parent-offspring communication, when either female parents or their offspring were inbred. I found that whilst inbred offspring begged less, parents provided inbred offspring with more care. The effects of inbreeding had significant consequences affecting biparental negotiation and parent-offspring communication. Next, I tested for the effects of inbreeding on the antimicrobial properties of secretions that both parents apply to the carcass during larval development. I found that the bactericidal activity of inbred male parents was reduced compared to outbred male parents during the dispersal stages and no evidence for the secretions of inbred and outbred female parents differing. Finally, to test whether the strong inbreeding depression found in this species influenced the mating decisions, I presented females with related or unrelated males, and found no evidence that females avoided inbreeding. These results show that to accurately estimate the fitness consequences of inbreeding the social effects on all individuals within a family must be accounted for.

Declaration

I declare that I have composed this thesis, under guidance from my supervisor. I conducted experimental work with help as detailed below, unless otherwise stated all work was my own.

Chapter 2 Data was collected by Luke Strutt to investigate inbreeding depression in juvenile and adult *N. vespillodies*. I performed all the analyses presented here and wrote the subsequent manuscript and chapter.

Chapter 5 Laboratory methods (secretion collection, lytic assay and bactericidal assay) were outlined by Dr Andreas Arce and Dr Daniel Rozen. I performed all experimental work, data collection, lab work, analyses and wrote the subsequent chapter.

This work has not been submitted for any other degree or professional qualification.

Sarah Mattey

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1 General Introduction

Inbreeding occurs in a wide range of plant and animal species, shaping the evolution of behaviours and matings systems, and posing a conservation risk to populations (Armbruster and Reed, 2005; Charlesworth and Willis, 2009; Crnokrak and Roff, 1999; Keller and Waller, 2002; Ralls et al., 1979). The selection pressures and extinction risks that inbreeding exerts on a population is therefore of interest to both evolutionary and conservation biologists. Furthermore, the effects of inbreeding can provide information on particular traits, providing evidence for their evolution and genetic architecture (Falconer and Mackay, 1996). Here, I test the effects of inbreeding on social behaviours associated with parental care, and use these effects to answer further questions on social traits and evolutionary conflict within a family.

1.1 Inbreeding Depression

1.1.1 Genetic Causes

Inbreeding is described at the individual level as when two mates share allele pairs that are identical by descent (Lynch and Walsh, 1998) and at the population level as when two mates have a higher relatedness to each other than they would if breeding at random (Crow and Kimura, 1970). When two related individuals mate, the probability they will both pass on alleles to their offspring that are identical by decent is increased (Falconer and Mackay, 1996). Inbreeding reduces the level of heterozygosity (Plomin et al., 2001), and this reduction is used as a measure for the level of inbreeding in Wrights' (1922) inbreeding coefficient, F. The inbreeding coefficient ranges from 0, a fully outbred population in Hardy-Weinberg equilibrium to 1, a fully inbred and homozygous population (Crow and Kimura, 1970).

Inbred individuals typically have lower fitness compared to outbred individuals, a phenomenon known as inbreeding depression (Lynch and Walsh, 1998). The genetic causes of inbreeding depression are relevant both to the practical concerns of

conservation and in answering evolutionary questions, such as the presence of genetic variation in fitness-related traits (Charlesworth and Willis, 2009). Two hypotheses are proposed to explain the phenomenon of inbreeding depression, each with different evolutionary implications (Lynch and Walsh, 1998). These hypotheses are not necessarily mutually exclusive and both rely on genetic dominance effects rather than purely additive genetic effects (Lynch and Walsh, 1998). First, the dominance hypothesis states that inbreeding increases the probability that two copies of rare deleterious recessive alleles will be inherited and expressed in offspring (Plomin et al., 2001). Second, the overdominance hypothesis states that heterozygosity leads to a higher fitness and vigour compared to both recessive and dominant homozygosity, and the decrease in heterozygosity as a result of inbreeding therefore reduces fitness and vigour (Lynch and Walsh, 1998). Currently, studies on both laboratory (Ayroles et al., 2009; Fernandez et al., 1995; Fox et al., 2008), and wild populations (Charlesworth and Willis, 2009) suggest that the dominance hypothesis accounts for the majority of inbreeding depression. Whilst the prevalence of overdominance effects is lower, overdominance still warrants further consideration when investigating inbreeding depression (Lynch and Walsh, 1998). The mechanisms by which inbreeding depression occurs affects both the severity and patterns of inbreeding depression for different traits. For example, the purging of deleterious alleles leading to the recovery of inbreeding depression is predicted only for the dominance hypothesis, whilst overdominance predicts that mean fitness cannot recover (Lynch and Walsh, 1998).

1.1.2 Traits Affected By Inbreeding Depression

Inbreeding depression is typically associated with a reduction in life-history and morphological traits (Falconer and Mackay, 1996), such as body size, development time, fecundity, vigour and survival (Crow and Kimura, 1970; DeRose and Roff, 1999). The effects of inbreeding have also been studied at the transcriptional level, showing that inbreeding affects genes associated with metabolic pathways, biosynthesis, oxidative stress and behaviour (Ayroles et al., 2009; Kristensen et al., 2006). The same traits tend to be affected by inbreeding depression within species and across populations, even between lab and wild populations (Fox et al., 2007). However the severity of inbreeding depression may vary between populations if their inbreeding histories differ (Pemberton, 2008). For example, if a population has a history of inbreeding, the deleterious effects are more likely to have been purged from the population and it would have less severe inbreeding depression compared to a population with no history of inbreeding. The severity of inbreeding depression depends on the species, populations and environments (Hedrick and Kalinowski, 2000; Saccheri et al., 1996).

Inbreeding depression is often reported to have particularly strong effects on fitness-related traits (DeRose and Roff, 1999; Falconer and Mackay, 1996; Lynch and Walsh, 1998). This is because inbreeding reduces the mean value of traits which are under directional dominance, and selection of these fitness related alleles is predominantly in one direction (Falconer and Mackay, 1996). If the alleles that increase the value of a trait are dominant over their alleles that reduce their value, then inbreeding will result in a change in the direction of the more recessive alleles and consequently lead to a reduction in the population mean in traits associated with fitness, such as life-history traits (Falconer and Mackay, 1996). In comparison, morphological traits are less associated with fitness and are predicted to be less affected by inbreeding depression. This is because either they are additive and have no dominance variation, because their dominance is not directional or because their dominance is less directional than fitness-related traits (DeRose and Roff, 1999). The genetic architecture of fitness related traits, including life-history traits, show that mutations affecting these traits are often recessive and highly deleterious, supporting that fitness related traits suffer the highest reductions as a consequence of inbreeding (DeRose and Roff, 1999; Falconer and Mackay, 1996; Kearsey and Kojima, 1967; Lynch and Walsh, 1998). However, some studies do report strong inbreeding depression in morphological traits. Inbred individuals in the social spider, *Stegodyphus lineatus*, have lower growth rates and body size than outbred individuals, but do not differ in survival and hatching success (Bilde et al., 2005). Overall, a meta-analysis on non-domestic animals supports the general trend that life-history traits show greater inbreeding depression than morphological traits, though some morphological traits are affected (DeRose and Roff, 1999). Variation in traits affected by inbreeding may be explained by the context of a trait, as some morphological traits are more closely related to fitness, such as those under differential sexual selection (Enders and Nunney, 2010; Mallet and Chippindale, 2011). This may reflect why a difference in traits affected by inbreeding is often reported (Enders and Nunney, 2010). This is because there is also variation between sexes in whether particular traits are related to fitness (Enders and Nunney, 2010; Mallet and Chippindale, 2011).

The study of inbreeding depression has often focussed on early life-stage traits such as hatching success and juvenile survival (Fernandez et al., 1995; Kempenaers et al., 1996). Inbreeding depression is often reported to be expressed most strongly in early life-stages (Husband and Schemske, 1996), but studies also show that inbreeding depression increases as more life-stages are measured (Kruuk et al., 2002; Mallet and Chippindale, 2011; Pusey and Wolf, 1996). It is now generally acknowledged that it is necessary to measure inbreeding depression over an entire life-cycle to accurately measure inbreeding depression (Charlesworth and Charlesworth, 1987) and in fact, several more recent studies have shown that inbreeding depression may be more severe in later life. For example, in the song sparrow, *Melospiza melodia*, inbreeding

depression is similar in adult compared to juvenile individuals (Keller, 1998). This increase in inbreeding depression in later life-stages is supported by a number of other studies (Costa e Silva et al., 2011; Keller et al., 2008; Winn et al., 2011), suggesting that inbreeding depression should not be assumed to be greatest in early life-stages (Winn et al., 2011). The investigation of fitness-related traits in later life-stages includes adult traits associated with mating, as these are closely related to fitness. Indeed, mating traits are commonly reduced by inbreeding. For example, in the field cricket *Telogryllus commodus*, the mating call time of inbred males is reduced (Drayton et al., 2010). Furthermore, male courtship is hampered in *Drosophila montana* (Aspi, 2000), male attractiveness is reduced in *D. simulans* (Okada et al., 2011) and *Tenebrio molitor* (Polkki et al., 2012), and mating ability and mating behaviours are significantly impaired in inbred *D. melanogaster* (Miller et al., 1993). Inbreeding also leads to reductions in sperm traits such as sperm number, precedence, offence and defence of inbred male *Tribolium casteneum* (Michalczyk et al., 2010) and oldfield mice, *Peromyscus polionotus* (Margulis and Walsh, 2002). Finally, female seed-feeding beetles *Callosobruchus maculatus* mated to inbred males have fewer sperm in their reproductive tract, despite that the ability of males to transfer sperm and the amount of sperm transferred are not being reduced by inbreeding. This reduction in sperm in the reproductive tract not only has fitness consequences for inbred males but also reduces the fecundity of outbred females mated to inbred males (Fox et al., 2012).

1.1.3 Indirect Social Effects of Inbreeding

While studies on social traits such as courtship and mating success demonstrate the potential fitness consequences of inbreeding for inbred individuals (Ilmonen et al., 2009; Okada et al., 2011), few have extended the fitness consequences to other individuals involved in the social behaviours (Fox et al., 2012; Vitikainen et al., 2011). Investigating the effects of inbreeding on social interactions by focussing only on one individual in the interaction ignores the response and hence the consequences of inbreeding for the other individual, potentially underestimating (or overestimating) the costs of inbreeding in a social group. Such effects of inbreeding may be described as indirect effects, i.e. effects of inbreeding that extend to individuals other than those that are inbred. In wood ants, *Formica exsecta*, the colony's productivity is reduced and haploid males are smaller when diploid female workers are inbred (Vitikainen et al., 2011). One form of social interaction that has been investigated in relation to indirect effects of inbreeding is between parents and offspring, in which inbred parents reduce the fitness of outbred offspring, a class of indirect effects described as intergenerational effects. Studies on wild populations of the song sparrow, *M. melodia*, Seychelles warbler *Acrophalus sechellensis* and the great tit, *Parus major*, find that

reductions in outbred offspring fitness are associated with increased inbreeding coefficients or homozygosity in female parents (Reid et al., 2003; Richardson et al., 2004; Szulkin et al., 2007). Intergenerational and other indirect effects of inbreeding within a family may be particularly important in species where parents provide elaborate parental care for their offspring. One context in which these indirect effects might be expected is where offspring fitness depends on a suite of parental traits such as provisioning of food and influencing the environment that offspring are be raised in (Clutton-Brock, 1991).

Parental care has been suggested as a mechanism for intergenerational effects of inbreeding in *M. melodia* and *P. major* (Reid et al., 2003; Szulkin et al., 2007). Parental care can be defined as any parental behaviour that increases offspring fitness, including survival and growth traits (Clutton-Brock, 1991). Parents invest a large amount of energy that might reduce an individual's condition, future reproductive attempts and future survival (Houston et al., 2005). There are many forms of parental care including investment of resources in gametes, care of eggs and provisioning of young (Clutton-Brock, 1991). Given that parental care is a major life-history trait that determines both parental and offspring fitness (Clutton-Brock, 1991; Royle et al., 2012), inbreeding might be expected to have strong effects on parental care. Milk production traits such as milk yield, protein and fat concentrations and length of time producing milk is reduced in inbred dairy cattle (Bjelland et al., 2013; Mc Parland et al., 2007; Smith et al., 1998; Thompson et al., 2000). In addition to dairy cattle, egg traits, such as egg number and hatching success, are reduced in a number of poultry species (Jull, 1933; Sewalem et al., 1999; Sittmann et al., 1966). Margulis (1998) investigated the social effects of inbreeding on parental care in two oldfield mice sub-species, *Peromyscus polionotus subgriseus* and *P. p. rhoadsi* and found that inbred female parents provided as much maternal care, the majority of care provided to offspring, as outbred female parents. However, inbred male parents did reduce the amount of care provided, but this did not lead to intergenerational effects on offspring (Margulis, 1998).

1.2 Inbreeding Avoidance

The significant fitness costs of inbreeding depression (DeRose and Roff, 1999; Drayton et al., 2010; Miller et al., 1993) are expected to result in the evolution of inbreeding avoidance (Blouin and Blouin, 1988). The relative costs of inbreeding and avoiding inbreeding determine whether inbreeding is avoided, tolerated or actively pursued in a population (Kokko and Ots, 2006). The evolution of such strategies is highly dependent on the context of the mating strategy of the organism (Kokko and Ots,

2006). There are several mechanisms that are proposed to have evolved to avoid the associated costs of inbreeding including mate choice, extra-pair copulations (EPCs) and sex-biased dispersal.

Mate choice is a commonly studied mechanism of inbreeding avoidance (Blouin and Blouin, 1988; Johnson et al., 2010; Kuriwada et al., 2011; Lihoreau et al., 2007; Pusey and Wolf, 1996; Thomas and Simmons, 2011; Tien et al., 2011), in which individuals prefer not to mate with kin (Blouin and Blouin, 1988). Many species are able recognise kin either based on familiarity (requiring advanced cognition), or by cues associated with relatedness (Lihoreau et al., 2007). Odours associated with the major histocompatibility complex (MHC) in vertebrates and cuticular hydrocarbons (CHCs) in insects are suggested to be used to recognise unfamiliar kin (Juola and Dearborn, 2011; Thomas and Simmons, 2011; Tregenza and Wedell, 2000). In addition, polyandrous females may choose to fertilise sperm of non-related males over related males (Bretman et al., 2009; Tregenza and Wedell, 2000, 2002). Browns' (1997) heterozygosity theory suggests that mate choice may be based on heterozygosity and compatible genes rather than 'good genes', thus leading to the avoidance of mating with close kin based on potential homozygosity. Support for this compatible genes hypothesis is found in female butterflies, *Colias*, which mate preferentially with more heterozygous males, though there is no evidence for increased fitness benefits from the resulting heterozygosity (Tregenza and Wedell, 2000). Hence, inbreeding avoidance by mate choice might not be through the selection of non-relatives, but may occur as a by-product of individuals choosing mates based on compatible genes.

Evidence for inbreeding avoidance by sex-biased dispersal and EPCs is mixed (Edly-Wright et al., 2007; Kempenaers, 2007; Pusey and Wolf, 1996; Stiver et al., 2008; Szulkin et al., 2012). If sex-biased dispersal is an inbreeding avoidance mechanism, theoretical models predict that inbreeding costs should be high and that females should be the dispersing sex because of the differences between the sexes in costs of inbreeding (Waser et al., 1986). However, whilst female birds do tend to be the dispersing sex, in mammals it is males that tend to disperse (Waser et al., 1986). A study on collared fly catchers, *Ficedula albicollis*, suggests that only close inbreeding will be avoided by sex-biased dispersal (Pärt, 1996). The authors also suggest that sex-biased dispersal in other populations may require strong inbreeding depression to benefit its evolution and the mechanism still presents difficulties in separating the cause of its evolution from other benefits such as intrasexual or resource competition (Pärt, 1996; Pusey and Wolf, 1996). For the avoidance of inbreeding by EPCs in communal breeders such as *Malurus splendens* and *M. elegans*, the risk of inbreeding is high, and there is evidence that EPCs with less related mates than female's social partners reduce the number of inbred offspring produced (Brooker et al., 1990; Brouwer et al., 2011). Studies on great tits *P. major* and house sparrows *Passer*

domesticus provide no evidence of avoiding inbreeding through EPC (Edly-Wright et al., 2007; Szulkin et al., 2012). In the blue tit, *Cyanistes caeruleus*, evidence suggests that in a population with low levels of inbreeding, EPCs are not used to avoid inbreeding (Kempenaers et al., 1996) whilst in another population, EPCs do lead to inbreeding avoidance (Foerster et al., 2003). Behaviours which reduce inbreeding may be difficult to determine as having evolved to avoid inbreeding as they may have been favoured by selection for other reasons such as to increase heterozygosity, leading to inbreeding avoidance as a consequence (Charlesworth and Charlesworth, 1987). The presence of inbreeding avoidance relates to inbreeding histories, costs of inbreeding, mating strategies and whether inbreeding exerts a strong selection pressure on a population.

1.3 Evolutionary Conflict Within The Family

Family interactions are strongly linked to fitness and are under strong selection because of the evolutionary conflict present within families over the partitioning of resources (Trivers, 1974). Thus, it may be hypothesised that inbreeding depression may be associated with parental care and offspring begging. Families are subject to several forms of evolutionary conflict, defined as when one trait that maximises the fitness of one individual does not maximise the fitness of other individuals (Lessells, 2012). Interacting individuals within a family are all in such a situation. Conflict occurs between male and female parents over how much each parent should invest in offspring, between parents and offspring over how much resources from the parents should be allocated to offspring, and between siblings over the amount of resources parents should invest in each offspring (Parker et al., 2002). To understand how these conflicts are resolved and how each influences the allocation of care the actual amount of investment by parents needs to be measured (Parker et al., 2002).

The conflict that occurs between two parents concerns two individuals that are typically unrelated (Lessells, 2012). Both parents benefit from the total investment by either parent, but only pay the cost of their own investment (Lessells, 2012). This results in each parent benefitting from their partner investing more than themselves (Lessells, 2012). The difference between the male's and female's optimum, or the how much the amount of care a parent provides is below its own optima, determines the extent of the conflict (Godfray, 1995; Lessells, 2006). Whether a parent provides the optimum amount of care will not only depend on the the focal parent but on the behaviour of their partner (McNamara et al., 1999). Theory offers two explanatory processes: a 'sealed bid' response (Houston and Davies, 1985) or negotiation over repeated bouts (McNamara et al., 1999). The Houston and Davies (1985) model

assumes a sealed bid process in which one bout of investment is provided by each parent and the amount of care provided by each parent is not re-negotiated in response to their partner's bid. However, evidence suggests that parents do respond to one another when deciding the amount of parental investment, and the negotiation model incorporates this to argue that decisions are made over repeated bouts of adjustment and interactions between parents (Houston et al., 2005; McNamara et al., 1999). The negotiation model predicts that after desertion of the brood or a reduction in the amount of care by one sex the other parent should increase the level of investment, thereby partially compensating for the partner reducing their investment (Houston et al., 2005; McNamara et al., 1999). Therefore the response by each parent to changes in the amount of care provided by their partner provides different predictions for the sealed bid and negotiation models.

The conflict between parents and offspring concerns two related individuals. As parental investment in offspring increases, so do the benefits for offspring, eventually reaching an asymptote. While the costs to parents increase with increasing parental investment, offspring suffer only half the costs of parental investment due to the relatedness asymmetry between parents and offspring (Mock and Parker, 1997). The difference in the costs of parental care between parents and offspring causes a conflict over the optimum parental investment (Mock and Parker, 1997). The offspring's amount of care is higher than the parents' optima, meaning that offspring benefit from more investment than parents benefit from providing (Mock and Parker, 1997). Two models of resolution have been proposed for parent-offspring conflict (Parker et al., 2002). The outcome of the scramble competition model is that 'offspring win' in receiving parental investment closer to their own optimum (Mock and Parker, 1997). This model suggests that investment by parents is determined by scramble competition between siblings, the winner of which receives higher investment (Mock and Parker, 1997). The outcome of the honest signalling model is that 'parents win' and their investment is closer to their own optimum. This model assumes that parents determine how much to invest by responding to honest signals of need by offspring, and invest more in hungrier offspring (Godfray, 1995; Mock and Parker, 1997). However, distinguishing these models has proved difficult as they make several similar predictions to one another (Royle et al., 2002b). Both models predict that solicitation by offspring incurs costs to the offspring, that parents should provision offspring based on offspring's signals and that offspring begging reflects need (Royle et al., 2002b). The two models may also be distinguished because the scramble competition model predicts that food allocation and begging costs are influenced by competitive asymmetries whilst honest signalling models predict that food is allocated in response to need, compensating for competitive asymmetries between siblings (Royle et al., 2002b). The conflict resolution is likely to lie on a continuum, where the level of investment will vary depending on biological context of parental investment (Parker

et al., 2002).

Theoretical predictions on parental care are based on the assumption that two parents providing care are unrelated or that parents and offspring share half their genes (Trivers, 1972). However, if genetic relatedness is increased between two individuals as a consequence of inbreeding this may potentially increase the benefits of cooperation and reduced competition between individuals. Thünken et al. (2007) show that an increase in relatedness between two related parents is associated with increased biparental cooperation over the provision of care. The benefits of inbreeding in terms of increased cooperation, and few apparent costs in terms of inbreeding depression, was also associated with active inbreeding in the species, suggesting a net benefit to inbreeding (Thünken et al., 2007). Therefore, the effects of inbreeding on behaviours associated with the resolution of evolutionary conflict, such as biparental cooperation, may be particularly important as the effects may determine the net costs or benefits of inbreeding. To date, there is little theory that predicts how the resolution of evolutionary conflict between family members is affected by inbreeding, though social evolution theory predicts increased relatedness facilitates the evolution of cooperation (Hamilton, 1964). In order to provide testable predictions on the resolution of family conflict, such theory should not only focus on interactions between parents, but also between parents and offspring and between related offspring within broods.

1.4 Study System: The Burying Beetle, *Nicrophorus vespilloides*

Burying beetles, (Silphidae: *Nicrophorus*), are an excellent study system for parental care because they raise offspring on a small vertebrate carcass on which they feed begging larvae pre-digested carrion (Figure 1.1 a and b). The interactions between parents and offspring of *Nicrophorus* have strong fitness implications for all family members and have been used to answer questions on cooperation and conflict within a family in field and laboratory populations (Scott, 1998). *Nicrophorus* comprises of 75 species mainly in the northern hemisphere and species from North America and Northern Europe. *Nicrophorus* have short life-cycles, allow large sample sizes and a controlled pedigree, and so a laboratory population is an ideal model for the study of social effects of inbreeding associated with parental care. Here, I use a European laboratory population of *N. vespilloides* which has a Paleartic distribution, reproducing in forest habitats (Scott, 1998). The parental care present in the species provides an opportunity to investigate how the presence of inbreeding depression influences the level and amount of a wide range of parental care traits.

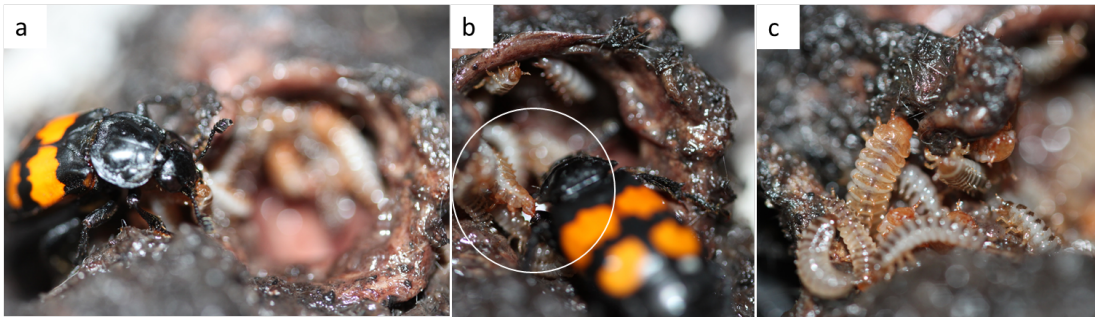

Figure 1.1: *Nicrophorus vespilloides* parent and offspring on a mouse carcass. (a) A female parent feeds a larva pre-digested carrion. (b) A larva attempts to solicit food from the female parent by tactile begging. (c) Larvae self-feed from the carcass.

1.4.1 Life-history

In *N. vespilloides*, male and female adults first find and mate on a carcass (day 1 of the breeding cycle). The carcass is then prepared by removing fur or feathers, after which the carcass is rolled into a ball, buried in the soil and a crater is created in the carcass from which the larvae later feed (Eggert and Müller, 1997). The female lays her eggs in the soil close by during days 2-3, whilst both parents continue to maintain and prepare the carcass (Eggert and Müller, 1997; Scott, 1998). The carcass stimulates the production of oral and anal secretions in male and females, which they apply to delay decomposition and protect the larvae from infection (Cotter and Kilner, 2010a; Eggert and Müller, 1997; Rozen et al., 2008; Scott, 1998). Once the eggs hatch, males often desert the carcass to find another mate (Scott, 1998; Scott and Gladstein, 1993). Larvae can be raised by either two, one, or no parents, and whilst offspring raised without parents have reduced survival, offspring raised by one parent do not suffer any reductions in final mass compared to two parents (Scott, 1998). At hatching (day 4-5) the larvae move from the soil to the crater on top of the carcass and begin self-feeding until day 10 (Eggert and Müller, 1997, Figure 1.1 c). In addition to self-feeding, larvae exhibit tactile begging to parents, who respond by feeding offspring pre-digested carrion (Eggert and Müller, 1997, Figure 1.1 a and b). The caring parents continue guarding and maintaining the carcass as the larvae develop (Eggert and Müller, 1997). The larvae beg less to the parent as they grow older and self-feed more (Eggert and Müller, 1997). Towards the end of development when the mass of larvae has increased by almost 90-fold, larvae begin to disperse from the carcass into the surrounding soil (day 9-11) (Eggert and Müller, 1997). In the soil larvae have a wandering phase (day 12-17), after which larvae begin pupation (day 18-24) (Eggert and Müller, 1997). Eventually pupae eclose into adults, and are sexually mature within 7 to 10 days after eclosion.

The costs of providing care and raising a brood, in particular larger broods, reduces a female's fecundity and future investment in reproductive attempts (Ward et al., 2009). The size of the carcass also affects a female's investment in a current brood, with females investing more in the current brood on a large carcass (Creighton et al., 2009). On a small carcass, both males and females are more likely to desert broods (Ward et al., 2009). The size of a female does not affect fecundity but size does affect the number and size of offspring the female can raise on the carcass (Rauter et al., 2010). Smaller females produce larger broods with smaller larvae that are more variable in size (Rauter et al., 2010). The size of the carcass also affects the number of offspring a female can raise (Ward et al., 2009), though females do not adjust the number of eggs laid to the size of the carcass and instead adjust the brood size by filial cannibalism once larvae have hatched and recently arrived at the carcass (Bartlett, 1987a; Scott, 1998). Offspring often hatch asynchronously, creating asymmetries between junior and senior offspring (Smiseth et al., 2007). As females age or their immune system is activated, females produce larger broods in which a larger proportion of the carcass is allocated to the brood (Cotter et al., 2010a; Creighton et al., 2009).

1.4.2 Mating behaviour

Adult males have two alternative mate finding tactics, either emitting pheromones in the absence of a carcass or finding a carcass, both of which are employed to attract females (Eggert, 1992). When employing the first strategy, males have a reproductive attempt without having to find a carcass first, though they must still attract females and will only mate with females once, and so sire fewer offspring without a carcass (Eggert, 1992; Eggert and Müller, 1997). The carcass is therefore an unpredictable but highly valuable resource, and subject to strong intra- and inter-specific competition (Eggert and Müller, 1997; Scott, 1998). Larger individuals are more successful in securing the carcass from other intra-specific competitors, and size is most reliably predicted by the pronotal width (Otronen, 1988; Safryn and Scott, 2000). Prior experience often has an effect on the outcome and investment in competition for a carcass, and individuals appear to measure their opponents, suggesting that the resulting dominant mating pair are not based purely on size (Otronen, 1990; Safryn and Scott, 2000). Losers of a competition for a carcass may sneak copulations (Scott, 1998), though dominant males mate more (on average 100 times a day) and sire a higher proportion of offspring (Müller and Eggert, 1989; Pettinger et al., 2011). Both sexes may mate multiply on the carcass with different individuals, and also repeat mating with the same individual (House et al., 2008). Studies have struggled to find any benefits to females or offspring due to polyandry in females (though there are benefits to males) (House et al., 2009, 2011). While females benefit from mating twice

with one male, no benefits to multiple mates have been found, and benefits after two matings with one male was also reduced (House et al., 2009, 2011). Repeated matings may however provide an opportunity for post-copulatory mate choice by females, and it is shown that in the absence of a carcass subordinate males sire a higher proportion of offspring relative to the number of matings (Pettinger et al., 2011).

The likelihood of inbreeding in wild populations, dispersal behaviours, distances and population structure of *N. vespilloides* is largely unknown. It is therefore difficult to estimate the history of inbreeding present in this species. Larvae disperse from the carcass into the soil to pupate and once eclosed, adults search for carcasses and mates by flight (Eggert and Müller, 1997), suggesting that it may be unlikely for close relatives to encounter one another. However, it is possible that related individuals encounter one another when aggregating on a carcass (Eggert and Müller, 1997; Scott, 1998). The likelihood of inbreeding will therefore depend on the occurrence of carcasses and the consequent competition for the resource, which may vary depending on the size and type of habitat for a given population (Eggert and Müller, 1997). Hence, without more information on dispersal patterns and the availability of a carcass in a given habitat it is not possible to reliably predict the likelihood of inbreeding and the history of inbreeding in *N. vespilloides*.

1.4.3 Parental Care

Parental care increases offspring growth and survival in *N. vespilloides* (Eggert et al., 1998). Both parents cooperate to prepare the carcass, protect it and the brood from predators and competitors, apply antimicrobial secretions to the carcass, and provision the larvae with pre-digested carrion (Arce et al., 2012; Eggert et al., 1998; Rozen et al., 2008; Smiseth et al., 2005). Care appears to be regulated by juvenile hormone (JH): JH increases and plateaus at sexual maturity, further increases rapidly upon finding a suitable carcass, and increases once again at oviposition, whilst female parents have an additional surge in JH at the time of larval hatching (Trumbo, 1997). Sexual division in care is present for both the amount and the type of care parents provides. Females spend more time provisioning food to larvae and stay on the carcass for a longer period of time than males (Eggert et al., 1998; Smiseth and Moore, 2002; Smiseth et al., 2005), whilst males spend more time maintaining the carcass (Smiseth et al., 2005). The longer females stay at the carcass, the more care they provide (Boncoraglio and Kilner, 2012). While males provide no benefits by provisioning food, they do provide benefits to offspring through their assistance in carcass burial and guarding against inter-specific competitors (Scott, 1990). If a female parent deserts the carcass, the male parent will partially compensate care and increase feeding and

antimicrobial care, whilst if the male deserts the brood, the female will remain caring at a similar level to that observed when the male parent is present (Rauter and Moore, 2004; Smiseth et al., 2005). Females appear to benefit from rearing a brood alone, as both female life-span and current brood size are increased when the female cares alone (Boncoraglio and Kilner, 2012).

Both male and female parents apply oral and anal secretions to the carcass which improves larval growth and reduces a parent's future reproductive success (Rozen et al., 2008). As in provisioning, sexual division is present for the production of secretions, as females have higher antibacterial activity than males and males compensate and increase levels of antibacterial activity when widowed (Cotter and Kilner, 2010b). Anal secretions contain a beetle lysozyme protein which has both lytic and bactericidal activity, increasing in activity from a minimal baseline level at egg laying to a peak level when larvae arrive at the carcass (Arce et al., 2012). The lysozyme benefits the larvae by significantly increasing their survival, but imposes a potential cost to the parents through a reduction in their personal immunity (Arce et al., 2012; Cotter et al., 2010a, 2013). Whilst parents produce secretions that protect larvae from immune challenges, larvae also actively secrete themselves, and larval secretions also have lysozyme activity which peaks as larvae arrive at the carcass (Arce et al., 2013).

1.4.4 Begging

Offspring signal hunger through begging, and parents spend more time providing care to offspring that beg more, particularly in bi-parental conditions (Rauter and Moore, 1999; Smiseth and Moore, 2002). Asynchronous hatching of eggs leads to an asymmetry in age and size between siblings. Junior larvae beg more than senior, but senior larvae are more successful in gaining access to the parent, suggesting that the resolution of parent-offspring conflict is mediated by honest signalling (Andrews and Smiseth, 2013; Rauter and Moore, 1999; Smiseth and Moore, 2007). There is no evidence for substantial energetic costs of offspring begging (Smiseth and Parker, 2008). However, costs of offspring begging are imposed through increased risk of infanticide, as offspring that beg more are at a higher risk of being targeted by parents for brood reduction (Andrews and Smiseth, 2013). Begging by larvae is stimulated by parent's CHCs and offspring are unable to distinguish between genetic or foster parents (Smiseth et al., 2010). Offspring begging is at a peak 24 hours after hatching, and by 72 hours the benefits to begging are reduced and it is more efficient for larvae to predominantly self-feed from the carcass (Smiseth et al., 2003). However, parents continue to respond to offspring begging and remain near to the brood after 72 hours,

suggesting that the termination of care is under offspring control (Smiseth et al., 2003). The presence of parents also exacerbates sibling competition, as individual larvae in larger broods receive less care than larvae in smaller broods (Smiseth et al., 2007). This latter finding supports scramble competition models (Smiseth et al., 2007). While there is contradicting evidence for both modes of conflict resolution in parent-offspring interactions, it is noted that the two are not mutually exclusive to one another (Parker et al., 2002). For example, the finding that parents tend to favour senior larvae over junior larvae suggests that allocation is under parental control (Smiseth and Moore, 2007). However, this latter evidence does not necessarily exclude the evidence that parental care exacerbates sibling competition (Smiseth et al., 2003), as parents may control the allocation, whilst offspring may have control over the cessation of parental care.

1.5 Aims and Outlines for Thesis

I aim to investigate the effects of inbreeding on several aspects of parent and offspring social traits in *N. vespilloides*, using an experimental laboratory population maintained at the University of Edinburgh. Laboratory populations have provided valuable understanding and insights into the underlying patterns of inbreeding depression (Hedrick and Kalinowski, 2000). For example, experimental designs are a simple and common method used to measure inbreeding depression by comparing mean values of inbred and outbred individuals, as a proportion change in fitness (Hedrick and Kalinowski, 2000; Lynch and Walsh, 1998):

$$\delta = \frac{\text{Mean outbred} - \text{Mean inbred}}{\text{Mean outbred}}$$

There is no prior information on inbreeding depression in *N. vespilloides* and first, I aim to investigate whether inbreeding depression is present in *N. vespilloides*, measuring growth and survival of inbred offspring (Chapter 2). In addition, I aim to test for the presence of intergenerational effects of inbreeding in outbred offspring raised by inbred parents (Chapter 2). I then aim to test whether inbred male and female parents reduce the amount of parental care provided, and measure the differences in the amount of care provided when a focal parent or their partner is inbred or outbred. The latter also aims to investigate the indirect effects of inbreeding between two socially interacting individuals, and will look at how the inbreeding status and care of a partner affects an individual's own care (Chapter 3). I extend the interest in familial conflict to test for effects of inbreeding on parent-offspring communication. In particular, I aim to investigate how parents and offspring respond to one another when inbred or outbred, and measure the amount of begging by

offspring and the response of the parent in the amount of care provided (Chapter 4). I then investigate the mechanistic basis of intergenerational effects of inbreeding on the social immunity of parents, investigating how inbreeding affects the antibacterial properties of male and female parent's secretions (Chapter 5). Finally, I test the presence of inbreeding avoidance to investigate the evolutionary response to strong effects of inbreeding. I focus on pre-copulatory mate choice, taking into account both mating strategies of this species by testing for inbreeding avoidance in both simultaneous and sequential mate choice (Chapter 6).

2 Direct and Intergenerational Effects of Inbreeding

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Abstract

Inbreeding depression is the reduction in fitness caused by mating between related individuals. Inbreeding is expected to cause a reduction in offspring fitness when the offspring themselves are inbred, but outbred individuals may also suffer a reduction in fitness when they dependent on care from inbred parents. At present, little is known about the significance of such intergenerational effects of inbreeding. Here, I report two experiments on the burying beetle *Nicrophorus vespilloides*, an insect with elaborate parental care, in which I investigated inbreeding depression in offspring when either the offspring themselves or their parents were inbred. I found substantial inbreeding depression when offspring were inbred, including reductions in hatching success of inbred eggs and survival of inbred offspring. I also found substantial inbreeding depression when parents were inbred, including reductions in hatching success of eggs produced by inbred parents and survival of outbred offspring that received care from inbred parents. The results suggest that intergenerational effects of inbreeding can have substantial fitness costs to offspring, and that future studies need to incorporate such costs in order to obtain accurate estimates of inbreeding depression.

2.1 Introduction

Inbreeding depression is the reduction in fitness of individuals that are produced by matings between related mates, and is caused mainly by the expression of deleterious recessive alleles associated with an increase in homozygosity (Charlesworth and Willis, 2009; Falconer and Mackay, 1996; Lynch and Walsh, 1998). The study of inbreeding depression is an important issue in evolutionary and conservation biology because it leads to selective pressures that shape mating systems and mating behaviours (Charlesworth and Willis, 2009), and because it poses a conservation concern to many wild populations (Armbruster and Reed, 2005; Crnokrak and Roff, 1999; Keller and Waller, 2002; Ralls et al., 1979). There is ample evidence for significant costs of inbreeding from studies on a wide range of taxa, including birds, mammals, insects and plants. These studies show that the effects of inbreeding may have a detrimental effect on both juvenile and adult traits, including hatching success, offspring survival, and the number of eggs laid by adult females (Keller, 1998; Noordwijk and Scharloo, 1981; Slate et al., 2000; Szulkin et al., 2007), which in turn suggests that accurate estimates of inbreeding need to measure fitness consequences across the entire life cycle of an individual. The finding that inbreeding has strong effects on adult traits suggests that inbreeding may affect the fitness of outbred offspring when their fitness is determined or influenced by their parents. If this is the case, accurate estimates of inbreeding depression also need to account for intergenerational effects of inbreeding in parents on offspring fitness. Unfortunately, there has so far been little attention to the potential significance of intergenerational effects of inbreeding in species where offspring fitness is determined or influenced by their parents.

Current studies on intergenerational effects of inbreeding have focused mainly on various mating systems of plants or on domestic livestock, and have shown that inbreeding coefficients of parents influence the fitness traits of progeny (Adamec et al., 2006; Bjelland et al., 2013; del Castillo, 1998; Helenurm and Schaal, 1996; Mc Parland et al., 2007; Riginos et al., 2007; Sewalem et al., 1999; Smith et al., 1998). There are far fewer studies of intergenerational effects in inbreeding in non-domestic animals, where the potential for such intergenerational effects may be particularly important in species where parents provide elaborate parental care for their offspring. For example, a study on oldfield mice found that inbreeding in parents led to a reduction in male (but not female) parental care, although inbreeding in parents had no effect on offspring fitness (Margulis, 1998). A recent study on red deer, *Cervus elaphus*, compared the effects of inbreeding in offspring traits when either the offspring themselves or the female parents were inbred. This study found that inbreeding in offspring was associated with a reduction in birth weight and first year survival, while the inbreeding in female parents had no effects on the same offspring traits. Thus, this

study suggests that intergenerational effects of inbreeding are weak compared to the direct effects of inbreeding when the offspring themselves are inbred (Walling et al., 2011). On the other hand, there is support for intergenerational effects of inbreeding from a study on song sparrows, *Melospiza melodia* and the Seychelles warbler *Acrophalus sechellensis* which find that reductions in outbred offspring fitness is associated with higher inbreeding coefficients or homozygosity in genetic female parents (Reid et al., 2003; Richardson et al., 2004). Furthermore, a study on great tits, *Parus major*, found that offspring of inbred parents had lower fledging success, recruitment into the breeding population, and lower reproductive success than offspring of outbred parents (Szulkin et al., 2007). These findings suggest that there might be intergenerational effects of inbreeding resulting from detrimental effects of inbreeding on the amount of parental care provided to offspring.

In order to improve our understanding of the potential significance of such intergenerational effects of inbreeding, it is now important to investigate inbreeding depression in offspring traits both when offspring themselves are inbred and when their parents are inbred. To this end, I used the burying beetle *Nicrophorus vespilloides* as a study system because this is an insect with highly elaborate parental care whereby one or both parents defend the carcass and the brood against conspecific competitors and microbial decomposers, and provision pre-digested carrion to the begging larvae (Arce et al., 2012; Eggert et al., 1998; Rozen et al., 2008; Smiseth et al., 2003). Parental care has a strong effect on offspring fitness by enhancing offspring survival and growth and speeding up the offspring's rate of development (Eggert et al., 1998; Smiseth et al., 2003). Using this species, I conducted two controlled experiments where the inbreeding status of either the offspring or the parents were manipulated through experimental breeding, allowing the estimation of inbreeding effects on offspring traits when either the offspring themselves were inbred or when their parents were inbred. I monitored effects of inbreeding in offspring and parents on offspring traits during different stages of the life cycle, including hatching success of eggs, survival and growth of larvae during the period when they received care from their parents, and after independence. In the experiment on intergenerational effects of inbreeding, I manipulated inbreeding in male and female parents to test for potential sex differences in the effects of inbreeding in parents on offspring fitness.

2.2 Materials and Methods

2.2.1 Study Species

Like all members of the genus *Nicrophorus*, *N. vespilloides* breeds on carcasses of small vertebrates, which are buried underground, rolled into a ball and then prepared and maintained by both parents (Eggert and Müller, 1997; Scott, 1998). Females lay eggs in the soil around the carcass over a mean period of 30 hours and the eggs hatch about 60 hours after laying (Smiseth et al., 2006). Once the larvae hatch, they crawl to the carcass, where they obtain food by self-feeding directly from the carcass and by begging for regurgitated food from the parents (Smiseth et al., 2003). Parents provide elaborate care that includes provisioning of food to the larvae, protecting and defending the brood from predators and competitors, and applying antimicrobials to defend the brood from microbes (Arce et al., 2012; Eggert et al., 1998; Rozen et al., 2008; Smiseth et al., 2003). Females are more involved in direct care for the larvae than males, and also provide care for a longer period of time (Eggert et al., 2008; Smiseth and Moore, 2002). The larvae disperse from the carcass about five to six days after hatching, pupate about 10 to 12 days after dispersal, and finally eclose as adults about 12 days after pupation.

2.2.2 General Methodology

Beetles were used from a large outbred laboratory population maintained at The University of Edinburgh. Inbreeding in the stock population was avoided by maintaining a large stock population and ensuring that breeding occurred among unrelated or distantly related individuals, defined as when two individuals did not share a common ancestor two generations or more back in time. The population comprised of 5th generation beetles from lines originally collected at Corstorphine Hill, Edinburgh, and 16th generation beetles from lines originally collected at Kennel Vale, Cornwall. Thus, we are confident that the stock population would not have been subject to any significant forms of inbreeding, purging or selection that would have biased the estimates of inbreeding depression in our experiments.

The beetles were maintained at 20°C under constant light conditions in transparent plastic containers (12cm x 8cm and 2cm high). Non-breeding adults were fed organic beef twice a week. Only virgin beetles were used for the experiments, and each individual was only mated once. Experimental pairs were bred in transparent plastic boxes (17cm x 12cm and 6cm high) by providing them with 1cm of moist soil and a

previously frozen mouse carcass with a mass of 20 ± 5g. The mice were supplied from Livefoods Direct Ltd, Sheffield, UK. The containers were checked for eggs on days 1-7 after pairing by noting the number of laid eggs and the number of unhatched eggs (i.e., decomposing eggs) that were visible through the base and sides of the transparent container. This method was used as it reliably estimates the number of eggs laid while at the same time reducing the risks of damaging eggs through handling, which might reduce hatching success (Monteith et al., 2012). The containers were checked for dispersing larvae on days 10 to 12 after pairing by noting whether the larvae had left the carcass. Once the larvae had dispersed, the date, the number of larvae that were alive, and the mass of the brood was recorded. The dispersed larvae were then placed in individual containers and left to pupate and eclose. The individual containers were again checked for eclosing adults 27 to 34 days after pairing.

2.2.3 Experimental Design

Experiment 1: Inbreeding in Offspring

To examine the effects of inbreeding when the offspring themselves were inbred, a block design similar to the one used by (Fox et al., 2008) was used. Each block comprised of eight beetles, four male and four female, derived from two unrelated families of the stock population (i.e., families that did not share a common grandparent). Each block produced two broods of inbred offspring by mating a female from each of the two families with her brother (i.e., full-sibling mating), and two broods of outbred offspring by mating a female from each of the two families with the unrelated male from the other family (Fig. 2.1a). Such a block design provides a powerful means for attributing variation in offspring trait values to inbreeding among offspring in a way that controls for potential confounding effects due to genetic differences among the different families used in the experiment (Fox et al., 2008). Controlling for genetic differences among families is highly advantageous in studies of inbreeding depression because families share genes that may affect the mean values of the traits that are potentially affected by inbreeding (Fox, 2005). Thus, the use of a blocked design ensures that the same set of alleles contributes to both inbred and outbred broods, and potential effects due to genetic differences among families can therefore be excluded (Fox, 2005). This design also ensures that all parents used in the experiment are outbred, such that any effect due to inbreeding would be caused by inbreeding among the offspring.

Fifteen blocks were setup in this experiment, consisting of 10 families of 16th generation beetles and 13 families of 5th generation beetles, yielding a total of 30

inbred broods and 30 outbred broods. Three inbred matings (in three separate blocks) were excluded because one of the parents died prior to egg laying. All males were removed from the breeding container seven days after pairing because this experiment was designed to test for effects of inbreeding in the offspring and their subsequent fitness. Previous studies show that that the extent to which males stay with the brood to provide care or desert is highly variable in this species (Smiseth and Moore, 2002), and that the removal of the male has no effect on offspring fitness (Smiseth et al., 2005). Thus, the male was removed to control for potential variation in male behaviour.

The effects of inbreeding in the offspring were measured on the following traits: (i) Number of eggs recorded as the number of eggs laid for each brood; (ii) Hatching success recorded by counting the number of unhatched eggs (i.e., decomposing eggs) visible through the base and sides of the container, subtracting this number from the total number of eggs laid to give the number of successfully hatching eggs, and then calculated relative to the total number of eggs laid; (iii) Time to dispersal recorded as the number of days from when the parents were paired up to when larvae dispersed from the carcass; (iv) Survival to dispersal recorded by counting the number of larvae dispersing from the carcass, and then calculated relative to the total number of eggs laid; (v) Size at dispersal recorded by weighing the whole brood to the nearest 0.1 mg, and dividing the brood mass by the number of larvae in the brood to yield a mean body mass per larva in the brood; (vi) Overall offspring survival recorded as the number of larvae surviving to dispersal from the carcass, and then calculated relative to the number of eggs laid to for a given brood; (vii) Survival to eclosion recorded as the number of larvae successfully eclosing relative to the number of larvae dispersing from the carcass for a given brood. For measures of survival (hatching success, survival to dispersal, overall offspring survival and survival to eclosion), I calculated mean values for each treatment based on the proportion of surviving offspring for each brood, whilst I used the number of surviving and non-surviving offspring as binomial variables in the statistical models (see below).

Experiment 2: Inbreeding in Parents

To test for possible intergenerational effects of inbreeding in parents on the offspring's fitness, a novel block design based on the inbred and outbred individuals generated from Experiment 1 was used. The design comprised of eight beetles derived from four unrelated families (i.e., families that did not share a common grandparent). Each block comprised two females from two different families (i.e., two sets of full sibling sisters), one of which was inbred and one of which was outbred, and two males from

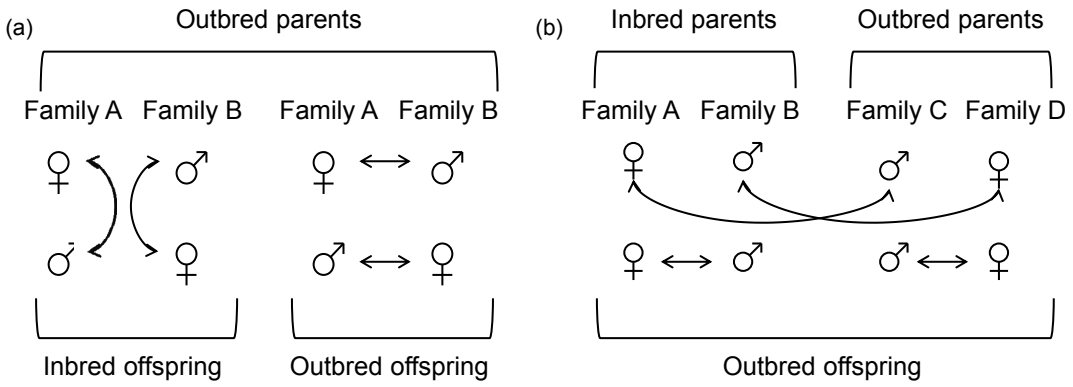

Figure 2.1: The two block designs used to test for the effects of inbreeding in offspring and parents in *Nicrophorus vespilloides*. (a) In Experiment 1, each block comprised four males and four females derived from two unrelated families of the stock population. Each block produced two broods of inbred offspring by mating a female from each of the two families with her brother (i.e., full-sibling mating), and two broods of outbred offspring by mating a female from each of the two families with the unrelated male from the other family. (b) In Experiment 2, each block comprised four males (two outbred and two inbred) and four females (two outbred and two inbred) derived from four unrelated families. Mating within each block was designed to ensure that all offspring were outbred, and that these offspring were produced and cared for by one of the following four treatment combinations: an inbred female mated to an inbred male; an inbred female mated to an outbred male; an outbred female mated to an inbred male; and an outbred female mated to an outbred male.

two additional families (i.e., two sets of full sibling brothers), one of which was inbred and one of which was outbred (Figure 2.1b). These beetles were mated to produce the following four treatments: (i) an inbred female mated to an inbred male; (ii) an inbred female mated to an outbred male; (iii) an outbred female mated to an inbred male; (iv) an outbred female mated to an outbred male (Figure 2.1b). Thus, this setup produced a two-by-two factorial design where parents of either sex were either inbred or outbred. The design allowed the comparison of the inbreeding effects in male and female parents on the offspring's fitness, while at the same time ensuring that all offspring were outbred (offspring were always outbred because all parents, inbred or outbred, were mated to an unrelated male or female (Falconer and Mackay, 1996; Frankham et al., 2002). Thus, any effects of inbreeding on offspring fitness in this experiment would be due to inbreeding among parents. Furthermore, this factorial design also allowed us to detect any potential differences in the effects of inbreeding in females and males. Such effects might be found in this species because female parents are more involved in direct care for the larvae than males (Eggert and Müller, 1997; Smiseth and Moore, 2002). Because I was interested in testing for such a sex difference, the male was not removed from the breeding container in this experiment, thus allowing both parents to provide parental care. Eleven blocks were created, yielding a total of 11 broods of each treatment with the exception of the treatment

comprising an inbred female and an outbred male, which was reduced to 10 broods because one adult died before mating. To determine whether inbreeding in parents had an effect on offspring traits, I estimated the same offspring and adult traits as in Experiment 1 (with the exception of time to dispersal and survival to eclosion, which was not recorded due to time limitations), following the same procedures as described above for Experiment 1.

2.2.4 Statistical Analysis

All data was analysed using R version 2.15. I used linear mixed-effects models for traits that had a normal error distribution (the number of eggs laid and size at dispersal in both experiments), and I used generalised linear mixed models for traits with a Poisson error structure (time to dispersal), and for traits with a binomial error structure (hatching success, survival to dispersal, overall offspring survival and survival to eclosion). All analyses were based on family means in order to avoid pseudoreplication due to obtaining data on multiple siblings. In Experiment 1, the experimental treatment (i.e., whether offspring were outbred or inbred) was assigned as a fixed effect, whilst in Experiment 2, the experimental treatments (i.e., whether males or females were outbred or inbred) were added as two fixed effects (i.e., one for each sex). In all models, block was assigned as a random factor. In the models of three traits (size at dispersal, survival to dispersal and survival to eclosion), I included additional covariates in the initial statistical models because these covariates were predicted to have an effect on the trait in question. Firstly, in the model of size at dispersal, I included brood size as an additional fixed effect because brood size is likely to determine the amount of resources available to each offspring and therefore the size of offspring (Smiseth et al., 2007). Secondly, in the model of survival to dispersal, I included mouse mass as an additional fixed effect because survival may be influenced by the amount of resources available to the brood. Thirdly, in the model of survival to eclosion, I added size at dispersal as an additional fixed effect because the size of the larvae may have a possible effect on the larvae's ability to eclose successfully. I based decisions as to whether to include these covariates in the final model based on AIC model selection criteria, according to which a covariate should be added if it significantly improves the fit of the model. In the analysis on survival to dispersal, which aimed to establish the effect of inbreeding on larval survival from hatching to the time of dispersal, I excluded two broods from Experiment 2 where no eggs hatched. In the analyses on inbreeding in parents (Experiment 2), I always first tested for a main effect of inbreeding depending on the sex of the parent before I generated separate models for the interaction between the inbreeding status of males and females. For all traits, I calculated inbreeding depression as a proportional change in

mean fitness of inbred (w_i) and outbred (w_o) individuals using the equation as $\delta = w_o - w_i/w_o$ (Hedrick and Kalinowski, 2000). Tables summarise the effect of inbreeding on each trait while controlling for the effects of block and other additional fixed effects listed above, while Figures shows means and standard errors for each trait based on the raw data. Thus, all inferences about the effects of inbreeding are based on the statistical analyses reported in the tables.

2.3 Results

2.3.1 Experiment 1: Inbreeding in Offspring

I first examined the additional covariates in the initial models to determine whether to include these in the final models on the effects of inbreeding on size at dispersal, survival to dispersal and survival to eclosion. Although larger broods produced larvae that were significantly smaller at dispersal ($t_4 0$ = - 5.491, $P < 0.001$), AIC model selection criteria suggested that brood size should not be included in the final model for size at dispersal. Given that these results indicate some ambiguity concerning the effect of brood size, I conducted two separate tests in which brood size was either included or excluded. These tests confirmed that the exclusion or inclusion of brood size had no effect on estimates of the effects of inbreeding, and I therefore report effects from a model in which brood size was excluded. Mouse mass had no significant effect on larval survival to dispersal ($Z = 0.3$, $P = 0.76$), and was not included in the final model on survival to dispersal based on AIC model selection. Finally, larvae that were larger at dispersal were more likely to survive to eclosion (Z = - 2.826, P = 0.005), and size at dispersal was included in the final model for survival to eclosion based on AIC model selection.

There was no significant difference in the number of eggs laid by females mated to a full-sib male and females mated to an unrelated male (Table 2.1). Thus, there was no evidence suggesting that females adjusted their reproductive investment in response to the risk of inbreeding in their offspring.

Inbreeding in offspring caused substantial inbreeding depression in a wide range of offspring traits (Figure 2.2; Table 2.1): such inbreeding was associated with reductions in hatching success of eggs by 3.5%, survival to dispersal by 8.5%, survival to eclosion as adults by 8.7% and overall offspring survival by 11.2% (Table 2.3). Inbreeding in offspring had a substantial effect on offspring survival both during the period when larvae depend on parental care (i.e., from egg laying to dispersal from the carcass) and

after independence (from dispersal to eclosion), corresponding to an overall reduction in offspring survival by 19.9%. In contrast, inbreeding had no significant effect on the time to dispersal and size at dispersal (Table 2.1), suggesting that inbreeding had no detectable effect on the duration of life-history stages or the body size of surviving offspring.

2.3.2 Experiment 2: Inbreeding in Parents

I first examined the additional covariates in the initial models to determine whether these should be included in the final models on the effects of inbreeding on the size at dispersal and survival to dispersal. As in Experiment 1, larger broods produced significantly smaller larvae at dispersal ($t_1 7$ = - 3.97=, $P = 0.001$), but in this experiment, brood size was included in the final model based on AIC model selection criteria. Mouse mass had no significant effect on the survival to dispersal (Z= -1.338, $P = 0.181$), and was not included in the final model of survival to dispersal based on AIC model selection.

Inbreeding in male and/or female parents caused substantial inbreeding depression in a wide range of offspring traits, including a reduction in hatching success of eggs, survival to dispersal and overall offspring survival (Table 2.2). As explained in detail further below, the intergenerational effects of inbreeding were often complex, involving for some traits differences in the effects of inbreeding in male and female parents and effects due to the interaction between inbreeding in the two sexes (Table 2.2). Inbreeding in males and females had no significant main effects on the number of eggs laid by females. However, there was a significant effect of the interaction between inbreeding in males and females, reflecting that inbred females increased the number of eggs they laid by 70% when mated to an inbred male as opposed to when they were mated to an outbred male. In contrast, outbred females laid a similar number of eggs regardless of whether they were mated to an inbred or outbred male (Figure 2.3a; Table 2.2).

Inbreeding in male and female parents had no significant main effects on hatching success of eggs (Table 2.2). However, hatching success was significantly influenced by the interaction between inbreeding in male and female parents (Table 2.2). This interaction effect occurred because hatching success of eggs sired by inbred males was decreased by 12% when mated to an inbred female as opposed to when mated to an outbred female, while hatching success of eggs sired by outbred males remained similar regardless of whether they were mated to an inbred or outbred female (Table 2.3). Inbreeding in female parents had a significant negative main effect on survival to

dispersal, whilst inbreeding in male parents had no such main effect (Table 2.2; Table 2.3). The significant main effect of inbreeding in females was associated with a significant effect of the interaction between inbreeding in males and females (Table 2.3). This interaction effect reflected that inbreeding in females reduced larval survival to dispersal by 38% when females were mated to an outbred male, while there was no effect of inbreeding in females when they were mated to an inbred male (Table 2.3). Inbreeding in female parents also had a significant negative main effect on survival to dispersal, whilst inbreeding in male parents had no such main effect (Figure 2.2; Table 2.3). However, there was no significant effect of the interaction between inbreeding in male and female parents on overall offspring survival (Table 2.2). Finally, inbreeding and males and females had no significant effect on the size of larvae at dispersal and there was no effect of the interaction between inbreeding in males and females on this trait (Table 2.2).

Table 2.1: Direct effects of inbreeding on offspring traits in *Nicrophorus vespilloides*. For each trait, there is information on parameter estimates (Par) with standard error (SE), test statistics (t-value for number of eggs and size at dispersal, and Z-value for time to dispersal, hatching success, survival to dispersal, overall offspring survival and survival to eclosion), and P values based on comparisons between outbred and inbred offspring. All data are provided by linear mixed-effects models of egg number and size at dispersal and generalised linear mixed models for time to dispersal (fitted with Poisson error structure), hatching success, survival to dispersal, overall offspring survival and survival to eclosion (fitted with a binomial error structure) in which experimental block is assigned as a random factor in all cases.

Trait	Par	SE	Z-Value	P-Value
Number of eggs	2.83	2.1	1.35	0.184
Hatching success (%)	-0.821	0.22	-3.83	0.0001
Time to dispersal (days)	0.014	0.081	0.18	0.855
Survival to dispersal (%)	-0.43	0.126	-3.42	0.0006
Size at dispersal (g)	-0.004	0.008	-0.462	0.647
Overall offspring survival (%)	-0.535	0.116	-4.611	$<$0.0001
Survival to eclosion (%)	-0.733	0.18	-4.12	$<$0.0001

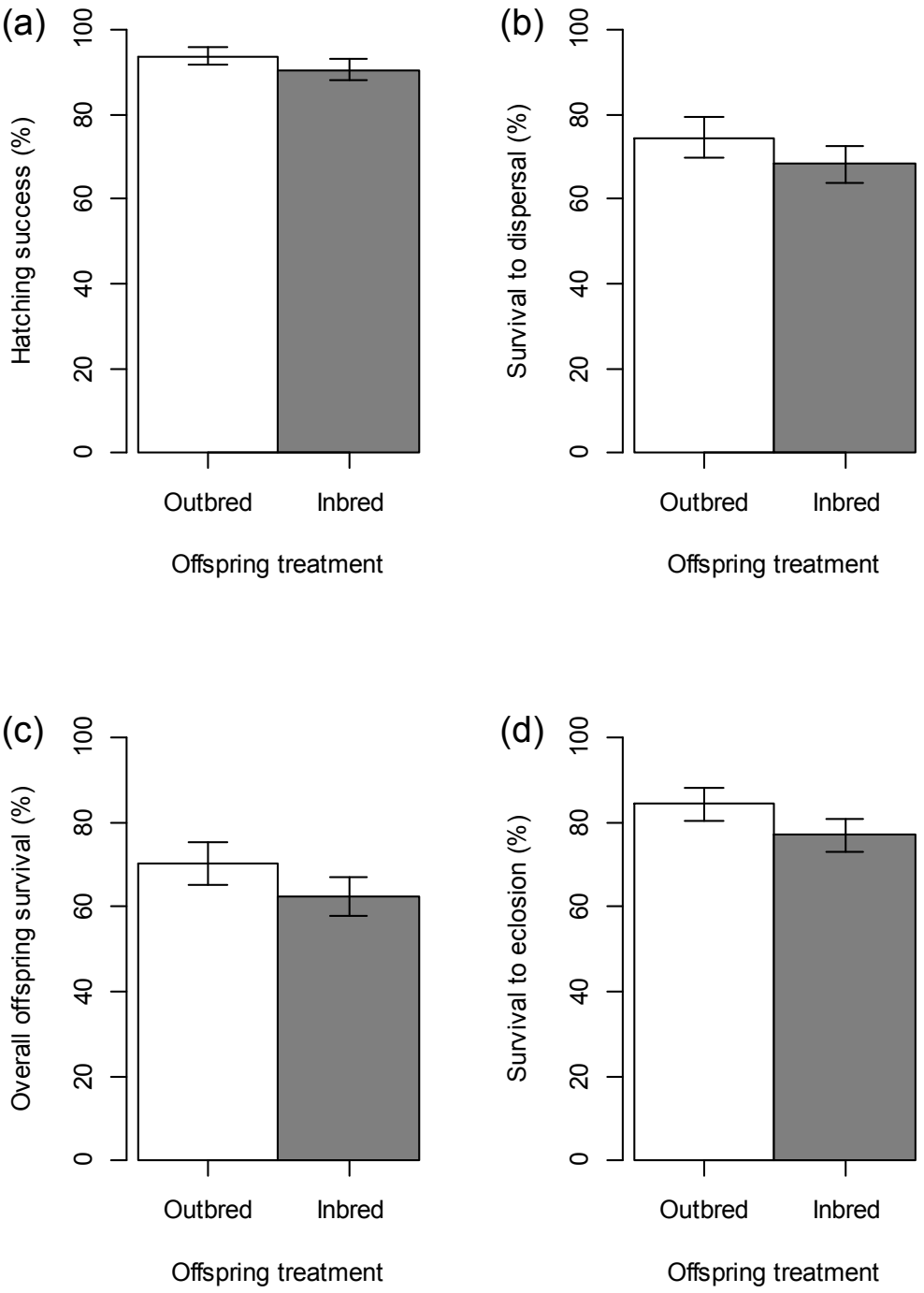

Figure 2.2: Direct effects of inbreeding on offspring traits in *Nicrophorus vespilloides*. Comparisons of hatching success (a), survival to dispersal (b), overall offspring survival (c), and survival to eclosion (d) when offspring were outbred (white bars) or inbred (grey bars) (mean ± SE).

Table 2.2: Intergenerational effects of inbreeding in parents on the traits of outbred offspring in *Nicrophorus vespilloides*. For each trait, I present parameter estimates (Par), standard error (SE), test statistics (t-value: number of eggs and size at dispersal and Z-value: hatching success, survival to dispersal and overall offspring survival, and P values based on comparisons of outbred offspring produced by and receiving care from males that were outbred or inbred, females that were outbred or inbred, and the effect of the interaction between inbreeding in males and females.

	Male				Female				Interaction			
Trait	Par	SE	t/Z Value	P Value	Par	SE	t/Z Value	P-Value	Par	SE	t/Z Value	P-Value
Number of eggs	7.09	2.66	2.66	0.01	-0.64	2.66	-0.239	0.812	11.93	5.04	2.37	0.0248
Hatching success (%)	-0.304	0.16	-1.881	0.06	-0.17	0.16	-1.069	0.285	-1.51	0.34	-4.431	<0.0001
Survival to dispersal (%)	-0.097	0.16	-0.61	0.541	-0.407	0.15	-2.67	0.0076	1.0	0.32	3.14	0.0017
Size at dispersal (g)	0.007	0.01	0.822	0.422	0.002	0.01	0.268	0.792	0.011	0.02	0.676	0.508
Overall offspring survival (%)	-0.219	0.13	-1.639	0.101	-0.311	0.13	-2.35	0.0187	0.088	0.27	0.328	0.743

Table 2.3: Inbreeding depression δ was calculated for all traits as $\delta = w_o - w_i/w_o$. Inbred offspring δ was estimated for the direct effects of inbreeding. Inbred male δ, female δ and δ parents was estimated as intergenerational effects in outbred offspring in male parents, female parents and in both parents. All values were calculated based on the same data set, but based on comparing different sets of treatments. Inbreeding depression of both parents was calculated by comparing trait values of the treatment in which both the male and female parents were inbred with the trait values of the treatment in which both the male and female parents were outbred.

		Male parent δ		Female parent δ		
Trait	Offspring δ	Female outbred	Female inbred	Male outbred	Male inbred	Both parents δ
Number of eggs	-0.092	-0.049	-0.702	0.264	-0.193	-0.252
Hatching success (%)	0.035	-0.067	0.022	0.036	0.117	0.057
Time to dispersal (days)	-0.015	-	-	-	-	-
Survival to dispersal (%)	0.085	0.236	-0.218	0.386	0.022	0.253
Size at dispersal (g)	0.018	-0.035	-0.084	0.035	-0.011	-0.047
Overall offspring survival (%)	0.112	0.138	-0.3	0.4	0.095	0.221
Survival to eclosion (%)	0.087	-	-	-	-	-

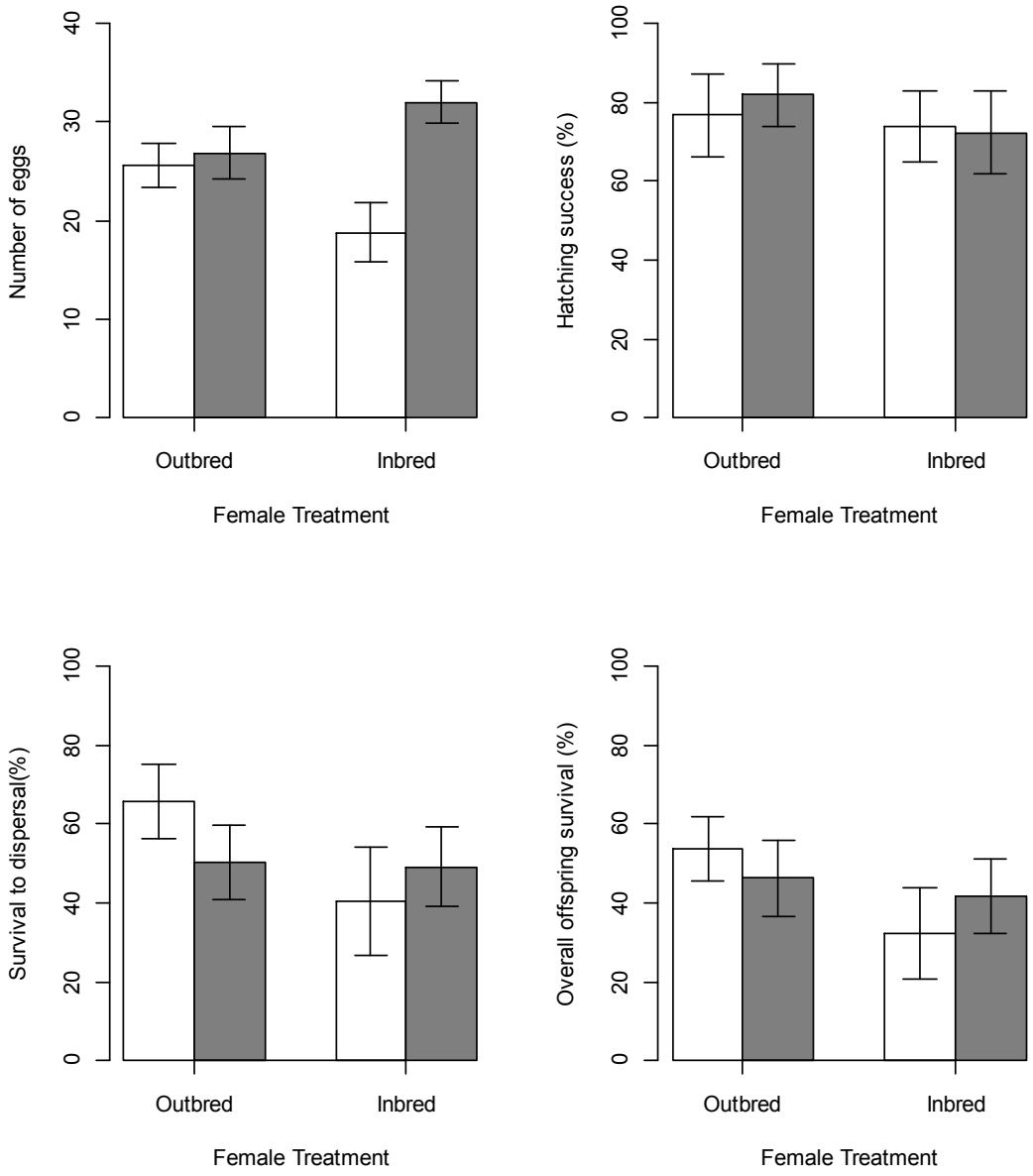

Figure 2.3: Intergenerational effects of inbreeding in parents on the traits of outbred offspring in *Nicrophorus vespilloides*. Comparison of the number of eggs laid (a), hatching success (b), survival to dispersal (c), and overall offspring survival (d) when outbred offspring were produced by and received care from outbred or inbred female parents (female treatment) and outbred (white bars) or inbred (grey bars) male parents (mean ± SE).

2.4 Discussion

Here, I report evidence from an experimental study on the burying beetle *N. vespilloides* demonstrating that inbred offspring suffer significant fitness costs and that outbred offspring also suffer significant fitness costs when their parents are inbred. The results provide a valuable contribution to the understanding of inbreeding

depression by providing a clear demonstration that offspring suffer the costs of inbreeding not only when they themselves are inbred but also when they are produced by and receive care from parents that are inbred. I argue that such intergenerational effects of inbreeding can be important in many species, particularly in species where parents provide elaborate care for their offspring, such as birds and mammals, and I suggest that further work on inbreeding in these species needs to consider the potential importance of intergenerational effects of inbreeding. Based on my results, I propose that the costs of inbreeding might extend to individuals other than those that are inbred, and that such indirect effects of inbreeding may occur in a wide range of social contexts in which the fitness of an outbred individual depends on the behaviour of individuals that are inbred. Below I provide a more detailed discussion of the major implications of the results.

The main aim of this study was to test for direct effects of inbreeding when the offspring themselves were inbred and intergenerational effects of inbreeding when outbred offspring were produced by and received care from inbred parents. I found evidence for substantial inbreeding depression in offspring traits when both the offspring themselves were inbred and when their parents were inbred. I found that the direct effects of inbreeding led to a reduction in overall offspring survival by 11% (Table 2.3). Meanwhile, intergenerational effects of inbreeding led to a reduction in overall offspring survival by 40% when females were inbred (and males were outbred), by 14% when males were inbred (and females were outbred) and by 22% when both parents were inbred (Table 2.3). These results demonstrate that inbreeding incurs substantial fitness costs in *N. vespilloides* due to both direct and intergenerational effects of inbreeding. In Experiment 1, males were removed from the brood to remove potential effects due to variation in male desertion, while males were left with the brood in Experiment 2 in order to provide estimates on the relative importance of inbreeding in males and females. Because of this difference in the design between the two experiments, caution is required when comparing the estimates of inbreeding depression when the offspring themselves were inbred and when their parents were inbred. For example, leaving males with the brood may have increased offspring fitness because males have the opportunity to provide additional care, but it may also reduce offspring fitness because males may start cannibalising larvae (Scott and Gladstein, 1993). Nevertheless, these results suggest that intergenerational effects of inbreeding may be comparable in strength, or even stronger, than the direct effects of inbreeding in *N. vespilloides*. This finding contrasts with a recent study on red deer, suggesting that the direct effects of inbreeding are much stronger than any intergenerational effects of inbreeding (Walling et al., 2011).

The finding that inbreeding had severe fitness costs to offspring when both they themselves were inbred and when the parents were inbred is somewhat surprising

because these effects must be based on different mechanisms. Although I did not specifically set out to investigate the mechanisms behind these effects, the results provide some suggestions for possible mechanisms based on observed reductions in survival during specific developmental stages. The observed reduction in hatching success and offspring mortality when the offspring (eggs or larvae) were inbred is likely to reflect mechanisms taking place after fertilisation, such as an increase in embryonic or larval mortality directly caused by the inheritance of rare homozygous deleterious recessive alleles. However, this mechanism cannot explain the reduction in hatching success or offspring survival when parents were inbred because in this situation the offspring (eggs or larvae) were outbred. Instead, the reduction in hatching success when parents were inbred must reflect mechanisms taking place prior to or during fertilisation, such as reduced quality or viability of eggs and sperm. Similar results have been reported in a recent study on the seed beetle, *Stator limbatus*, in which eggs laid by inbred females were found to be less likely to hatch (Messina et al., 2013). Finally, the reduction in offspring survival from hatching to dispersal when parents were inbred is likely to reflect mechanisms taking place after hatching, such as reduced quantity or quality of care provided by inbred parents.

I found evidence for a sex difference in the intergenerational effects of inbreeding. Firstly, inbreeding in males was associated with an increase in the number of eggs laid by an inbred female, whilst inbreeding had no such effect in females. Although this finding is consistent with females attempting to compensate for an expected reduction in hatching success when mated to an inbred male by increasing the number of eggs laid, I believe that such a conclusion is not warranted at this stage because I have not been able to replicate this finding in later experiments based on the same design (S.N. Mattey and P.T. Smiseth, unpublished data). Secondly, inbreeding in females was associated with a reduction in larval survival from hatching to dispersal and overall offspring survival, whilst inbreeding in males had no such effect. These results are consistent with females being more involved in direct care of larvae than males in *N. vespilloides* (Smiseth and Moore, 2002; Smiseth et al., 2005). Thus, the finding that inbreeding in females had a stronger impact on larval survival from hatching to dispersal may simply reflect that females are more involved in the form of care that is likely to have the strongest impact on larval survival; that is, the provisioning of food to larvae (Eggert et al., 1998; Smiseth et al., 2003).

I also found a significant interaction effect between inbreeding in males and females on the number of eggs laid, hatching success of eggs, and larval survival from hatching to dispersal. These interaction effects suggest that the intergenerational effects of inbreeding in a parent of a given sex may depend not only on its own inbreeding status but also on the inbreeding status of its partner. These interaction effects are likely to depend on different mechanisms. For example, the interaction effect on

hatching success may reflect that successful fertilisation depends on the quality of both sperm and eggs (both of which may be reduced by inbreeding), though further work is needed to confirm whether this is indeed the case. In contrast, the interaction effect on larval survival from hatching to dispersal occurs during the period where larvae receive care from their parents, and may potentially reflect that inbreeding alters the behavioural dynamics between male and female parents. Such effects might be expected in species with biparental care because each parent is expected to adjust its contribution towards care based on the amount of care provided by its partner (Houston et al., 2005; Lessells and McNamara, 2012). These considerations suggest that intergenerational effects of inbreeding may alter the complex behavioural dynamics of interactions among family members, and further work is now needed to investigate this intriguing possibility.

Although the finding that outbred offspring suffered from reduced survival when cared for by inbred parents suggests that inbred parents provide less care than outbred parents, it is important to recognise that other mechanisms such as reductions in maternal immunity might account for this finding (Reid et al., 2003). Nevertheless, our study provides some indications that inbreeding may affect the amount of care provided by parents. Firstly, inbreeding in parents had the strongest effect on offspring fitness during the period from hatching to dispersal. This represents the stage in the offspring's life cycle during which parents provide care for offspring (Eggert et al., 1998; Smiseth et al., 2003). Secondly, inbreeding in female parents was more detrimental for survival to dispersal than inbreeding in male parents, which is consistent with females being more engaged in direct parental care for larvae than males in this species (Smiseth and Moore, 2002). At present, surprisingly few studies have documented effects of inbreeding on parental care, particularly in non-domestic animals. Several studies show that inbred dairy cattle suffer reductions in several milk production traits such as milk yields and protein and fat concentrations (Bjelland et al., 2013; Mc Parland et al., 2007; Smith et al., 1998; Thompson et al., 2000), whilst a number of poultry species have reduced egg numbers and hatching success (Jull, 1933; Sewalem et al., 1999; Sittmann et al., 1966). A notable exception in non-domestic animals is a study on oldfield mice, which showed that inbreeding led to a reduction in the amount of care provided by males (but not by females), although in this case inbreeding in parents had no effect on offspring survival (Margulis, 1998). Thus, further work is needed to examine the potential effects of inbreeding on parental care.

The finding that intergenerational effects of inbreeding in parents can have strong and a detrimental effect on the offspring's fitness has important implications for the study of inbreeding. Firstly, if intergenerational costs of inbreeding are commonplace, ignoring such costs would run the risk of underestimating the true cost of inbreeding.

This might be particularly important to studies that seek to estimate inbreeding depression in species with elaborate forms of parental care, such as birds and mammals. Secondly, intergenerational effects of inbreeding may be part of a larger class of inbreeding effects that occur in a wide range of social contexts. These contexts include all instances where the fitness of an outbred individual depends on behavioural interactions with others and where inbreeding influences the behaviour of the individuals with which the outbred individual interacts. If so, such effects of inbreeding may be considered a form of indirect genetic effects, which occur when a trait expressed in a focal individual is influenced by genes in another individual with which the focal individual interacts (Moore et al., 1997). However, it should be noted that, in the case of inbreeding, these indirect genetic effects are based on dominance effects rather than on additive genetic effects as suggested for classical indirect genetic effects. Thus, I encourage further work on inbreeding to examine indirect effects in a wide range of social contexts, including competition, social dominance and aggressive interactions, and communal and cooperative breeding.

3 Effects of Inbreeding on Biparental Cooperation

Abstract

There is mounting evidence that inbreeding can have detrimental effects on the fitness of outbred individuals that interact with or depend on inbred individuals. However, little is currently known about the behavioral mechanisms by which interactions with inbred individuals induce fitness costs in outbred individuals. Here, I study effects of inbreeding on the behavioral dynamics of biparental cooperation in the burying beetle *Nicrophorus vespilloides*. To this end, I used a two-by-two factorial design, in which an inbred or outbred female was mated to an inbred or an outbred male and tested for effects on cooperation between male and female parents providing care for their joint offspring. I found no evidence that inbred parents provided less care than outbred parents. Nevertheless, partners of inbred parents increased the amount of care they provided, leading to overcompensation. These results show that inbreeding can have strong and complex effects on the behavioral dynamics of biparental cooperation, and that these effects are mediated mainly through changes in the partner's behavior. I suggest that similar effects of inbreeding on outbred individuals may extend to other social contexts, such as cooperative breeding and mating.

3.1 Introduction

Inbreeding is the result of matings between related individuals, and has attracted interest in evolutionary and conservation biology because it often leads to a reduction in fitness, commonly known as inbreeding depression (Crow and Kimura, 1970). Inbreeding depression is thought to result from an increase in homozygosity, which in turn, increases the risk that rare deleterious recessive alleles are expressed (Charlesworth and Willis, 2009; Falconer and Mackay, 1996; Lynch and Walsh, 1998). There is ample evidence that inbred individuals suffer fitness costs in terms of reduced growth, survival and reproduction (DeRose and Roff, 1999; Drayton et al., 2010; Miller et al., 1993). However, there is also mounting evidence that the detrimental effects of inbreeding can extend to outbred individuals that interact with or depend on individuals that are inbred. For example, the fitness of outbred offspring is often reduced when produced by inbred parents (Adamec et al., 2006; Bjelland et al., 2013; Mattey et al., 2013; Mc Parland et al., 2007; Reid et al., 2003; Richardson et al., 2004; Sewalem et al., 1999; Smith et al., 1998; Szulkin et al., 2007, Chapter 2). Furthermore, studies on beetles and flies show that females have reduced fecundity when they mate with inbred males (Fox et al., 2012; Okada et al., 2011), while a study on ants shows that the colony's productivity is reduced when the workers are inbred (Vitikainen et al., 2011). However, although these studies show that outbred individuals often pay a fitness cost from social interactions with inbred individuals, there are few studies on the behavioral mechanisms mediating such between-individual effects of inbreeding.

Biparental cooperation between male and female parents providing care for their joint offspring provides an excellent system for studying how between-individual effects of inbreeding are mediated through social interactions. First, it is likely that outbred parents suffer fitness costs from cooperating with an inbred partner (Mattey et al., 2013, Chapter 2) given that the offspring's fitness depends on the total amount of care provided by the two parents (Lessells, 2012). Second, theoretical models and empirical studies on the evolutionary stability of biparental care have established a good understanding of the behavioral mechanisms underlying this form of cooperation (Lessells, 2012). In species with biparental care, there is sexual conflict over the amount of care each parent should contribute because the benefit of care to each parent depends on the two parents' combined effort, while the cost depends only on each parent's personal effort (Lessells, 2012). The resolution of this conflict may be mediated through a negotiation process, whereby each parent adjusts its level of care in direct response to its partner's contribution (McNamara et al., 1999), or a 'sealed bid' process, whereby each parent makes an initial fixed decision about how much care to provide (Houston and Davies, 1985). Negotiation models predict that, if one parent reduces its contribution to care, its partner should respond by increasing its own

contribution, but only such that it compensates incompletely for this reduction (McNamara et al., 1999). In contrast, sealed bid models predict that the partner will not compensate for any reductions in the contribution by the other parent, although the partner could alter its initial fixed decision (Houston and Davies, 1985). The effect of inbreeding on the behavioral dynamics of biparental care therefore depends on the mechanism of conflict resolution. If sexual conflict is resolved through a negotiation process, it is expected that inbreeding affects biparental cooperation if inbred parents reduce their contribution to care, thereby causing incomplete compensation by their partner. Meanwhile, if sexual conflict is resolved through a sealed bid process, I expect effects of inbreeding if inbred parents reduce their contribution to care without triggering a compensatory response by the partner, or if partners of inbred parents alter their initial fixed decision in a way that is independent of the amount of care provided by inbred parents. The latter may occur if partners of inbred parents adjust their initial fixed decision in response to a cue from the other parent that is linked with inbreeding, such as its overall condition.

Here, I investigate the effects of inbreeding on biparental cooperation in the burying beetle *N. vespilloides*. This species is an excellent study system because it is one of the few insects where both parents provide care for the offspring (Eggert et al., 1998; Smiseth and Moore, 2004a). Like all members of the genus *Nicrophorus*, *N. vespilloides* breeds on the carcasses of small vertebrates (Scott, 1998). Both parents cooperate to prepare the carcass, protect it and the brood from predators and conspecifics, apply antimicrobials to the carcass, and provision the larvae with pre-digested carrion (Arce et al., 2012; Eggert et al., 1998; Rozen et al., 2008; Smiseth et al., 2005). Females spend more time provisioning food for the larvae and stay on the carcass for a longer period of time than males (Eggert et al., 1998; Smiseth and Moore, 2002; Smiseth et al., 2005), whilst males spend more time maintaining the carcass (Smiseth et al., 2005). There is evidence that parents adjust their contribution to the amount of care provided by the other parent (Smiseth and Moore, 2004a), although a removal experiment found that only males compensate (incompletely) when their partner is experimentally removed (Smiseth et al., 2005). A recent study found that outbred offspring suffered a reduction in survival when they received care from inbred parents, and that there was a significant effect of the interaction between inbreeding in males and females on larval survival (Mattey et al., 2013, Chapter 2). However, whilst evidence shows that inbreeding affects aggression and cognition (Bashi, 1977; Eklund, 1996), there is currently no information on the behavioral mechanisms whereby inbreeding affects biparental cooperation.

To test for effects of inbreeding on biparental cooperation, I used a two-by-two factorial design in which I paired an inbred or an outbred male to an inbred or an outbred female. All pairs were provided with an unrelated experimental brood that

comprised of 10 outbred offspring. This experimental design allowed us to test for differences in the amount of care provided by inbred and outbred parents as well as to test for differences in the amount provided by partners of inbred and outbred parents. In my analyses, I refer to the parent whose inbreeding status (inbred or outbred) was treated as the source of any effects as the 'focal parent', and the individual paired with the focal parent as its 'partner'. First, I tested for effects of the focal parent's inbreeding status on the amount of care it provided. I predict that inbred parents should provide less care than outbred parents given that parental care is costly and inbreeding should reduce an individual's ability to perform costly behaviors (DeRose and Roff, 1999; Falconer and Mackay, 1996; Lynch and Walsh, 1998). Second, I tested for effects of the focal parent's inbreeding status on the amount of care provided by its partner. If inbred parents provide less care than outbred parents, I predict that partners of inbred parents should increase their contribution and thus provide more care than partners of outbred parents. If this response is mediated through a negotiation process, I predict that any differences in the amount of care provided by partners of inbred or outbred parents should be explained by variation in the amount care provided by inbred and outbred parents. Furthermore, negotiation models predict that biparental care is evolutionary stable only if the partner responds by incomplete compensation (McNamara et al., 1999). To test this prediction, I compared the total amount of care provided by the two parents across the different treatment groups. Meanwhile, if sexual conflict is resolved through a sealed bid process, I predict that any differences in the amount of care by partners of inbred parents should be independent of variation in the amount of care provided by inbred and outbred focal parents. Third, this design allowed us to test for effects of the interaction between the inbreeding status of the focal parent and its partner. I predict that such interaction effects might be expected if inbred partners of inbred parents are more constrained in their ability to compensate than outbred partners of inbred parents. Finally, I tested for effects of inbreeding on the growth and survival of the offspring, which were always outbred in this experiment. Based on previous results from the same species (Mattey et al., 2013, Chapter 2), I predict that offspring have lower survival when they receive care from inbred female parents partnered with outbred males.

3.2 Material and Methods

3.2.1 General Methods

All beetles were maintained under laboratory conditions at 20^{o}C and under constant light. Non-breeding adults were housed individually in transparent plastic containers

(12cm x 8cm and 2cm high) and fed organic beef twice a week. Experimental pairs were bred in transparent plastic boxes (17cm x 12cm and 6cm high) by providing them with 1cm of moist soil and a previously frozen mouse carcass (supplied from Livefoods Direct Ltd, Sheffield, UK) with a mass range of 25 ± 2.5g. All beetles were from a large outbred laboratory population maintained at The University of Edinburgh. The stock population was bred to ensure that it was outbred and not subject to purging or selection that might bias the estimates of inbreeding from this experiment. To this end, I mated pairs that were not closely related in the sense that they did not share a common grandparent (or a closer relative). The stock population comprised of beetles originally collected at Corstorphine Hill and The Hermitage of Braid, Edinburgh; Jodrell Bank, Manchester; Kennel Vale, Cornwall; and Madingley Woods, Cambridge. I only used virgin beetles in the experiments. The outbred individuals used in the experiments were bred according to the same protocol as that for the stock population, while inbred individuals derived from matings between two full siblings. Once adult, both inbred and outbred individuals were maintained under standard laboratory conditions.

3.2.2 Experimental Design

To investigate the effects of inbreeding on the dynamics of biparental care, I used a blocked two-by-two factorial breeding design in which parents of each sex were either inbred or outbred (Mattey et al., 2013, Chapter 2). The experimental pairs were set up in 18 blocks, each of which comprised of four pairs (one from each treatment), thus yielding a total of 72 broods. I ensured that all experimental pairs comprised of individuals that were not closely related (i.e., did not share a common grandparent or a closer relative). I excluded 13 broods from further analyses because I did not have sufficient number of larvae to generate a brood, one brood because one of the parents died, and one brood because the larvae were hidden underneath the carcass, thus preventing the collection of accurate data on the amount of care provided by the parents. I note that this latter brood was included in my analyses on offspring growth and survival. Thus, the final sample sizes across the four treatment groups were as follows: (i) an outbred male mated to an outbred female (n=16); (ii) an outbred male mated to an inbred female (n=15); (iii) an inbred male mated to an outbred female (n=14); and (iv) an inbred male mated to an inbred female (n=12).

Immediately prior to hatching, I moved the parents and the prepared carcass to a new breeding box with fresh soil. Once the eggs had started hatching, I began generating experimental broods of ten by mixing larvae that had hatched from eggs laid by different females from across all treatments. I used this design because it excludes any

potential effects of inbreeding on the behavioral dynamics between the two parents mediated through the larvae's begging behavior or the number of larvae in the brood. Thus, the cross-fostering design effectively ensures that any effects of the focal parent's inbreeding status on its partner's behavior can be attributed to the interaction between the two parents. I also note that, because all experimental pairs comprised of individuals that were not closely related, the larvae that were used to setup the broods were always outbred. Given that the eggs hatch asynchronously over a mean period of about 30 hours (Müller and Eggert, 1990; Smiseth et al., 2006), I continued to generate experimental broods over a period of 2-3 days. I recorded the initial brood mass as this was used to calculate offspring growth from hatching to subsequent stages of larval development. Because parents will kill all larvae that arrive before their own eggs hatch (Müller and Eggert, 1990), I only provided a given pair with an experimental brood once their own eggs had hatched.

I conducted behavioral observations of the amount of care provided by inbred and outbred parents 24 hours after they had been provided with an experimental brood. I did the observation at this stage in the offspring's development because it corresponds to the peak in parental care in *N. vespilloides* (Smiseth et al., 2007). I recorded behavioral data using instantaneous sampling every 1 min for 30 min in accordance with established protocols (Smiseth and Moore, 2002). I recorded the number of scans each parent spent providing (1) direct care, defined as when the parent was provisioning food to the larvae (i.e., engaging in mouth-to-mouth contact with at least one larva) or interacting with the larvae (i.e., standing still and allowing larvae to beg); and (2) indirect care, defined as when the parent was guarding the carcass (i.e., standing still in the position where it could defend the brood from predators and interspecific competitors) or maintaining the carcass (i.e., depositing anal or oral secretions to the surface of the carcass, manipulating the carcass from below or excavating the crypt). I excluded time spent consuming carrion from my definition of direct care, because I often observed that males (but not females) would consume carrion without later provisioning food to the larvae. This observation suggests that males often consumed carrion to replenish their own energy reserves rather than to provision food for the larvae. Thus, my definition of direct care is slightly different to that used in a previous study on the same species (Walling et al., 2008).

Immediately after the observation, I counted the number of surviving larvae in the brood and weighed the brood to record the total brood mass. I used this information to determine larval growth and survival until the time of observation (i.e., 24 h after hatching). Once counted and weighed, I returned the larvae to the carcass. Parents were then allowed to raise the brood undisturbed until the larvae dispersed (approximately 8-10 days after hatching) from the carcass, at which point I recorded the number of larvae and weighed the brood to record the total brood mass. I used

Table 3.1: Design of statistical models to test for effects of inbreeding on biparental cooperation. In all models I always included the main effects of inbreeding status (IS) of the focal parent and its partner. Note that male IS represents the focal parent's IS and female IS represents the partner's IS for male (M) behaviours, while female IS represents the focal parent's IS and male IS represents the partner's IS for female (F) behaviours. For all parental care models brood size was also added as an additional effect. For each model, decisions on whether or not to include interaction effects and additional effects were based on the lowest AIC score. The order of terms in each column represents the order by which each term was added in the models. Block was assigned as a random effect in all models

Model	Main effect	Interaction	Additional effects
M direct care	Male IS + Female IS	Male IS * Female IS	F direct care
M indirect care	Male IS + Female IS	Male IS * Female IS	F indirect care
F direct care	Female IS + Male IS	Female IS * Male IS	M direct care
F indirect care	Female IS + Male IS	Female IS * Male IS	M indirect care
Total direct care	Male IS + Female IS	Male IS * Female IS	Brood size
Total indirect care	Male IS + Female IS	Male IS * Female IS	Brood size
Offspring growth	Male IS + Female IS	Male IS * Female IS	Brood size
Offspring survival	Male IS + Female IS	Male IS * Female IS	Carcass size

this information to measure larval growth and survival until the time of dispersal. After dispersal, I separated the larvae into individual containers to allow them to pupate and eclose as adults. Once the pupae had eclosed, I counted the number of surviving adults to measure survival to eclosion.

3.2.3 Statistical Analysis

I analyzed the data using R version 2.13. I used linear mixed-effects models (lme) for traits that had a normal error distribution (body mass of larvae at hatching, at the time of observation and at dispersal) and generalized linear mixed models (lmer) for traits that had a Poisson error structure (direct care and indirect care) or a binomial error structure (larval survival to observation, survival to dispersal and survival to eclosion). I assigned block as a random factor in all models. For parents of each sex, I built separate mixed effects models for effects of inbreeding on the time it spent providing direct and indirect care, and for effects of inbreeding on body mass of larvae at hatching, at the time of observation and at dispersal, and larval survival until the time of observation, at dispersal and at eclosion (Table 3.1).

I conducted separate models for each sex concerning the amount of direct and indirect care provided by inbred and outbred parents. In all such models, I always assigned the focal parent's inbreeding status (inbred or outbred) as the first main fixed effect, and

this term was always included in the final models (Table 3.1). I also always assigned the partner's inbreeding status (inbred or outbred) as the second main fixed effect (Table 3.1), and this term was also included in all final models. I next added the interaction term between the focal parent's inbreeding status and that of its partner (Table 3.1). The inclusion of the interaction effect was based on the lowest AIC score. I used the simplest model whenever the difference in the AIC score was less than two. In all these models, I also tested for effects of brood size on the amount of care provided by female and male parents (Table 3.1). Brood size was added as an additional fixed effect because, although I always provided parents with 10 larvae, there was some variation between broods in the number of larvae that were alive at the time of the observation. I found that females spent more time providing direct care (Z = 1.98, $P = 0.048$), and indirect care (Z = 2.02, $P = 0.043$) when caring for a larger brood, while there were no significant effects of brood size on male care (direct care by males: Z = -0.54, $P = 0.59$; indirect care by males: Z = -0.86, $P = 0.39$). The inclusion of additional fixed effects in final models was based on the lowest AIC score. I used the simplest model whenever the difference in the AIC score was less than two. Thus, I included brood size in the final model for direct and indirect care by females, while I excluded it from all male care models.

In order to assess whether any response by partners of inbred parents was mediated through a negotiation process, I conducted additional analyses by comparing models where the amount of time that the focal parent spent providing care was either added or removed as an additional fixed effect. If any such responses were mediated through a negotiation process, I predict that any differences in the amount of care provided by partners of inbred or outbred parents should be explained by variation in the amount care provided by inbred and outbred parents. In other words, if any such responses were mediated through a negotiation process, I expected that including the focal parent's behavior should remove or reduce the effect of the focal parent's inbreeding status on the amount of care provided by the partner. I also conducted separate analyses to examine the level of compensation. I conducted separate analyses for the total amount of direct and indirect care provided by both parents. These models included the inbreeding status of males and females as main fixed effects as well as the interaction between the two (Table 3.1).

For models on growth and survival, I always assigned the male's inbreeding status as the first main fixed effect and the female's inbreeding status as the second main fixed effect. These main fixed effects were included in all final models. I then added the interaction term between the inbreeding status of male and female parents. Finally, I added the additional fixed effects of carcass size (for survival traits) or brood size (for growth traits) (Table 3.1). The inclusion of the interaction effects and additional fixed effects was based on the lowest AIC score. I used the simplest model whenever the

difference in the AIC score was less than two.

3.3 Results

3.3.1 Effect on Focal Parent

I found no evidence that inbred parents provided less care than outbred parents (Table 4.3: Focal parent's IS; Figure 3.1a). Inbred females spent a similar amount of time providing direct and indirect care as outbred females (Figure 3.1a). Inbred males spent significantly more time providing indirect care than outbred males, while there was no significant difference in the amount of time spent providing direct care between inbred and outbred males (Figure 3.1).

3.3.2 Response by the Partner

There were significant differences in the amount of care provided by partners of inbred and outbred parents (Table 3.2: Partner's IS; Figure 3.1). Male partners of inbred females spent significantly more time providing direct care than male partners of outbred females, and female partners of inbred males spent significantly more time providing direct care than female partners of outbred males (Figure 3.1a). Female partners of inbred males spent significantly less time providing indirect care than female partners of outbred males, while there was no significant difference in the time spent providing indirect care by male partners of inbred or outbred females (Figure 3.1b). Thus, partners of inbred parents increased the amount of direct care they provided despite the fact that there was no difference in the amount of direct care provided by inbred and outbred parents (Figure 3.1a). As a consequence, the total amount of direct care provided by the two parents was higher when at least one parent was inbred than when both parents were outbred (Table 3.2); Figure 3.1a), indicating that partners of inbred parents responded by overcompensation rather than by incomplete or no compensation. Concerning indirect care, there was evidence for complete compensation as the total amount of indirect care was similar regardless of whether parents were inbred or outbred (Figure 3.1b).

In both sexes, the amount of direct care provided by the focal parent had a highly significant effect on the amount of direct care provided by partners (Table 3.2: Partner's behavior). This finding suggests that each parent adjusted its contribution to care in response to the amount of care provided by the other parent, as predicted

by negotiation models. To determine whether such a negotiation process accounted for the effect of the inbreeding status of the focal parent on the amount of care provided by its partner, I compared models in which I either included or excluded the amount of care provided by the focal parent as an additional fixed effect. I found that including or excluding the focal parent's behavior did not alter the effect that the focal parent's status (inbred or outbred) had on the amount of direct or indirect care provided by the partner. Indeed, partners of inbred parents still spent significantly more time providing direct care than partners of outbred parents if I excluded the focal parent's behavior in the models (male direct care: Z = 6.09, $P < 0.0001$; female direct care: Z = 3.36, $P = 0.0008$). Female partners of inbred males also spent significantly less time providing indirect care than female partners of outbred males when the male's behavior was included in the model (Z = -2.94, $P = 0.003$), and there was still no significant difference in the time spent providing indirect care by male partners of inbred or outbred females when the female's behavior was included in the model (Z = 0.091, $P = 0.93$). These results suggest that the effect of inbreeding on biparental care was not mediated through a negotiation process. Instead, the partners' response was independent of the actual amount of care provided by inbred or outbred parents, thus suggesting that this effect was mediated through a sealed bid process.

Table 3.2: Summary of statistical tests for the effects of inbreeding on male (M) and female (F) care in *Nicrophorus vespilloides*. Parameter estimates (Par), standard errors (SE), Z-values, and *P*-values for the effects of the focal parent's own inbreeding status (IS), that of its partner, their interaction and the partner's care.

	Focal IS				Partner's IS				Interaction				Partner's Care			
Trait	Par	SE	Z-Value	P-Value	Par	SE	Z-Value	P-Value	Par	SE	Z-Value	P-Value	Par	SE	Z-Value	P-Value
M Direct	-0.36	0.3	-1.21	0.23	1.27	0.33	3.9	<0.0001	-0.14	0.69	-0.2	0.84	-0.37	0.09	-3.95	<0.0001
M Indirect	0.47	0.19	2.44	0.015	0.02	0.19	0.12	0.9	0.79	0.39	2.05	0.041	-0.02	0.03	-0.6	0.55
F Direct	-0.0002	0.18	-0.001	0.99	0.67	0.16	4.08	<0.0001	-1.26	0.38	-3.41	0.0008	-0.54	0.17	-3.19	0.001
F Indirect	-0.04	0.15	-0.23	0.82	-0.5	0.16	-3.06	0.002	-0.92	0.36	-2.56	0.01	-0.013	0.03	-0.437	0.66

Table 3.3: Effects of inbreeding in two parents on outbred offspring growth in *Nicrophorus vespilloides*. Parameter estimates (Par), standard errors (SE), *t*-values, and *P*-values for the effects of the male's inbreeding status (IS), the female's IS, their the interaction and brood size.

	Male				Female				Interaction				Brood			
Trait	Par	SE	Z-Value	*P*-Value	Par	SE	Z-Value	*P*-Value	Par	SE	Z-Value	*P*-Value	Par	SE	Z-Value	*P*-Value
Hatching	$3x10^{-5}$	$7.9x10^{-5}$	0.44	0.66	$4x10^{-5}$	$7.9x10^{-5}$	0.53	0.6	$-4.6x10^{-5}$	$1.6x10^{-4}$	-0.29	0.78	-	-	-	-
Observation	0.001	0.0008	1.24	0.22	0.0001	0.0008	0.16	0.87	0.001	0.002	0.66	0.52	0.0003	0.0002	1.34	0.19
Dispersal	0.01	0.08	0.17	0.1	0.01	0.01	1.44	0.16	0.02	0.02	1.25	0.22	0.004	0.002	2.24	0.03

Table 3.4: Effects of inbreeding in two parents on outbred offspring survival in *Nicrophorus vespilloides*. Parameter estimates (Par), standard errors (SE), Z-values, and *P*-values test the effect of the male's inbreeding status (IS), the female's IS, their interaction and carcass size.

	Male IS				Female IS				Interaction				Carcass			
Trait	Par	SE	Z-Value	*P*-Value	Par	SE	Z-Value	*P*-Value	Par	SE	Z-Value	*P*-Value	Par	SE	Z-Value	*P*-Value
Observation	-0.17	0.21	-0.84	0.40	-0.22	0.21	-1.063	0.29	-0.69	0.44	-1.59	0.11	-0.5	0.21	-2.32	0.02
Dispersal	0.28	0.23	1.19	0.23	0.42	0.24	1.75	0.08	-0.24	0.50	-0.47	0.64	-0.53	0.22	-2.42	0.016
Eclosion	-0.4	0.36	-1.17	0.24	0.05	0.35	0.14	0.88	0.96	0.77	1.25	0.21	1.29	0.4	0.59	0.0013

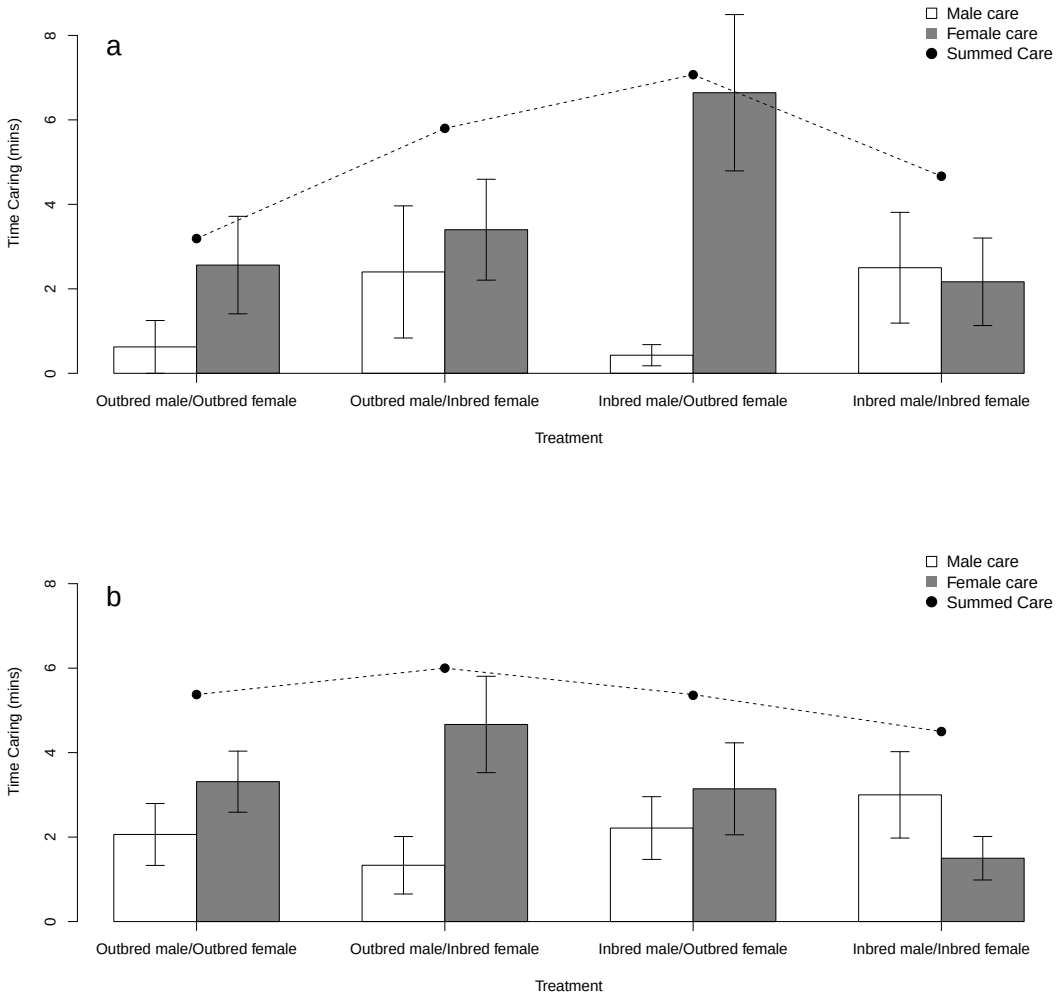

Figure 3.1: Effects of inbreeding on biparental cooperation in *Nicrophorus vespilloides*. Comparison of the amount of time spent providing direct care (a) and indirect care (b) by inbred or outbred male (white bars) and inbred and outbred female (grey bars) parents caring for outbred offspring during a 30 min observation (mean ± SE). The total amount of care offspring received from both parents (filled circles) is shown for each treatment (mean).

3.3.3 Interaction Between Inbreeding in Focal Parent and its Partner

The experimental design allowed us to test for effects of the interaction between inbreeding in female and male parents. I found that the interaction had a significant effect on the amount of care provided by both females and males (Table 3.2). The significant interaction effects for females reflected the fact that outbred females spent more time providing direct care when they were mated to an inbred male, whilst inbred females provided a similar amount of care regardless of whether they were

mated to an outbred or an inbred male (Figure 3.1a). Furthermore, inbred females spent more time providing indirect care when they were mated to an outbred male, whilst outbred females provided a similar amount of indirect care regardless of whether they were mated to an inbred or outbred male (Figure 3.1b). The significant interaction effect for males reflected that inbred males spent more time providing indirect care when they were mated to an inbred female, whilst outbred males provided a similar amount of indirect care regardless of whether they were mated to an inbred or outbred female (Figure 3.1b).

Table 3.5: Effects of inbreeding on total care provided by two parents in *Nicrophorus vespilloides*. Parameter estimates (Par), standard errors (SE), Z-values, and *P*-values for the effects of the female's inbreeding status, that of the male and the interaction (M*F) between the two are provided.

	Male				Female				(M*F)			
Trait	Par	SE	Z-Value	*P*-Value	Par	SE	Z-Value	*P*-Value	Par	SE	Z-Value	*P*-Value
Direct	0.31	0.12	2.57	0.01	0.23	0.13	1.77	0.07	-0.99	0.26	-3.78	0.0002
Indirect	-0.11	0.12	-0.95	0.34	-0.02	0.12	-0.13	0.898	-0.23	0.24	-0.959	0.337

3.3.4 Larval Growth and Survival

Finally, I tested for the effects of inbreeding in parents on the growth and survival of their outbred offspring. In contrast to what I predicted, I found no evidence of intergenerational effects of inbreeding in parents on offspring fitness (Table 3.3; Table 3.4). However, there were strong effects of brood size on larval growth and of carcass size on larval survival (Table 3.4). These effects reflected the fact that larvae grew better in smaller broods and that they survived better on larger carcasses.

3.4 Discussion

Here, I present evidence that inbreeding can have complex effects on cooperation among male and female parents caring for their joint offspring. I found that inbred parents provided as much direct care as outbred parents, but that partners of inbred parents nevertheless provided more direct care than partners of outbred parents. As a consequence, the total amount of direct care provided by the two parents was greater when at least one parent was inbred than when both parents were outbred. Although I found that partners of inbred and outbred parents responded to the actual amount of care provided by the focal parent, I found no evidence that the difference in care between partners of inbred and outbred parents could be explained by variation in the

amount of care provided by inbred and outbred focal parents. I also found effects of the interaction between inbreeding in males and females. Outbred females provided more direct care when mated to an inbred male, whilst inbred females provided a similar amount of direct care regardless of whether they were mated to an outbred or an inbred male. Given that biparental care is taxonomically widespread and the predominant pattern of care in birds (Clutton-Brock, 1991; Royle et al., 2012), my results highlight the need for further work to advance the understanding of potential effects of inbreeding in species with biparental care. Below I discuss the wider implications of my results in more detail.

I found that inbred parents provided as much, or more, care than outbred parents. My results therefore contradict the prediction that inbred parents should provide less care than outbred parents. My results also differ somewhat from those from a previous study on oldfield mice, *Peromyscus polionotus*, in which inbred males were found to provide less care than outbred males, although inbred females provided as much care as outbred females (Margulis, 1998). There are two reasons why inbred parents might be expected to provide less care than outbred ones. Firstly, inbreeding depression is often reported to have particularly strong effects on fitness-related traits, including life-history traits, reflecting that mutations affecting these traits often are recessive and highly deleterious (DeRose and Roff, 1999; Lynch and Walsh, 1998). Thus, given that parental care is a major life-history trait that determines both parental and offspring fitness (Clutton-Brock, 1991; Royle et al., 2012), inbreeding might be expected to have strong effects on parental care. Secondly, parental care often incurs significant fitness costs to parents (Clutton-Brock, 1991; Royle et al., 2012). Thus, given that inbreeding often causes physiological damage, inbreeding may reduce an individual's ability to cope with the energetic costs of parental care (Ayroles et al., 2009; Kristensen et al., 2006). My results call into question whether these two scenarios are met, at least for *N. vespilloides*. However, I argue that this seems unlikely given that previous work on this and closely related species suggests that parental care is costly and strongly linked with parental and offspring fitness (Cotter and Kilner, 2010b; Creighton et al., 2009; Eggert et al., 1998; Smiseth et al., 2003; Trumbo, 2007). Instead, I propose that inbreeding may have little or no net effect on parental care if any increase in the energetic costs of providing parental care is counteracted by a reduction in the opportunity costs through future matings (Kokko and Jennions, 2008). Inbred parents might have lower opportunity costs of care if they have a lower future reproductive potential than outbred ones (Aspi, 2000; Drayton et al., 2010; Miller et al., 1993). This scenario might also provide an explanation for inbred males provided more care than outbred males. In *N. vespilloides*, males normally provide less care than females, suggesting that males have higher opportunity costs of care than females, presumably because they can gain some reproductive success by mating with other females away from carcasses (Müller and Eggert, 1989). If so, inbreeding might have a stronger

effect on the opportunity costs of males, thereby causing inbred males to increase their contribution towards care for their current brood. These considerations suggest that there is now a need for further work to examine whether inbreeding has differential effects on the energy and opportunity costs of care in the two sexes.

My results show that partners of inbred parents provided more direct care than partners of outbred parents, while female partners of inbred males spent less time providing indirect care than female partners of outbred males. These findings are surprising given that I predicted such effects only if inbred parents provided less care, causing a subsequent compensatory response by the partner. My results show that partners of inbred parents increased the amount of direct care they provided despite that inbred parents provided as much direct care as outbred parents. As a consequence, there was an increase in the amount of total direct care when at least one of the parents was inbred (Table 3.5; Figure 3.1). Thus, partners of inbred parents responded by overcompensation rather than by incomplete or no compensation as I predicted. Previous studies on species with biparental care provide little evidence for overcompensation, although it has been reported in a small number of mate removal experiments on birds (Griggio and Pilastro, 2007; Royle et al., 2002a). Theoretical considerations suggest that there should be strong selection against overcompensation, as it allows the other parent to exploit its partner, thereby rendering biparental care evolutionarily unstable (Lessells, 2012; Lessells and McNamara, 2012). My evidence for overcompensation contradicts previous work on *Nicrophorus* beetles. Mate removal experiments report that males respond by incomplete compensation to female removal, while females show no compensation in response to male removal (Rauter and Moore, 2004; Smiseth et al., 2005; Suzuki and Nagano, 2009). Meanwhile, handicapping experiments show that, although handicapped parents provide less care, there is no compensation by the partner (Suzuki and Nagano, 2009). Further work is needed to examine why partner responses seemingly lead to overcompensation when a partner interacts with an inbred parent but not when a partner interacts with a handicapped parent or when the other parent is removed. One potential explanation for these diverging partner responses is that inbred parents provide lower-quality care (e.g., effectiveness per unit time devoted to care), while handicapped parents reduce their quantity of care (e.g., overall amount of time devoted to care). An alternative explanation is that the partners of inbred parents respond to changes in the phenotypic condition of inbred parents, while partners of handicapped parents do not respond specifically to changes in the amount of care by handicapped parents.

I also found that the focal parent's inbreeding status had a significant effect on the amount of care provided by the partner regardless of whether or not the amount of care provided by inbred and outbred focal parent was included in the model. If the partner's response to the focal parent's status was mediated through a negotiation

process, I expected that the inclusion of the amount of care provided by the focal parent in models on the care provided by the partner would either remove or reduce the effect of the focal parent's inbreeding status. Thus, my results provide no support for the suggestion that the effect of inbreeding on the behavioral dynamics of biparental care was mediated through a negotiation process. In contrast, my results show that partners of inbred parents increased their level of care independently of variation in the amount care provided by inbred and outbred parents, thus suggesting that this effect was mediated through a sealed bid process. The finding that partners of inbred parents altered their contribution of care independently of the amount of care provided by the focal parent suggests that partners of inbred parents must respond to some unknown cue that differed between inbred and outbred parents. This study provides no information as what the potential nature of such a cue might be, but one potential candidate is chemical cues, such as cuticular hydrocarbons (CHCs) (Howard and Blomquist, 2005). Such cues could vary between inbred and outbred parents either as a direct consequence of inbreeding or as an indirect consequence of correlated changes in other traits such as overall condition. There is some support for the suggestion from studies showing that females can discriminate between inbred and outbred males, some of which are based on chemical cues (Ilmonen et al., 2009; Okada et al., 2011; Zajitschek and Brooks, 2010). Furthermore, previous work on *N. vespilloides* shows that CHCs play an important role in partner recognition between pairs that cooperate to provide care for their joint offspring (Müller et al., 2003; Steiger et al., 2009). Further work is now needed to test whether CHCs or other chemical cues can provide direct or indirect information about inbreeding status in *N. vespilloides*, and whether such cues play a role in mediating partner responses to inbreeding in the context of biparental care.

I also found effects of the interaction between the inbreeding status of the focal parent and that of its partner on the amount of care provided by parents. I expected such interactions effects to arise if inbred partners of inbred parents were more constrained in their ability to compensate than outbred partners of inbred parents. If so, inbred partners should provide less care overall and be less responsive to the other parent's inbreeding status than outbred partners. In contrast, my results show that inbred females provided as much direct and indirect care as outbred females. Furthermore, although inbred partners were less responsive to the focal parent's inbreeding status than outbred partners with respect to direct care, inbred partners were more responsive to the focal parent's inbreeding status with respect to indirect care. I am unaware of any alternative explanations from the literature that might explain the specific patterns of these interaction effects. However, I note that, in my experimental design, the amount of care provided that a given parent provides is influenced by both its own inbreeding status and that of its partner. Thus, inbred partners might provide less direct care when mated to inbred parents than when mated to outbred parents

because they also responded to the inbreeding status of their partner. For example, given that inbred males provided more care when mated to inbred females than when mated to outbred females, inbred females may have been able to reduce their direct care when mated to inbred males. These considerations highlight that these interaction effects reflect the outcome of complex dynamics between decisions made by either sex as a result of their own inbreeding status, the inbreeding status of their partner and the amount of care provided by the partner. In order to improve the understanding of the dynamics of biparental care, I argue the use of my fully factorial design should be extended to handicapping experiments, in which a focal parent's ability to provide care is manipulated by for example adding weights to the individual, thereby increasing energetic costs of providing care (Harrison et al., 2009). So far, handicapping experiments have targeted only one parent, either the male or the female (Harrison et al., 2009), and it is therefore unclear whether interaction effects are limited to the context of inbreeding or whether they also occur when contributions to parental care are manipulated by handicapping.

Finally, I found no evidence that inbreeding in male or female parents had detrimental effects on the offspring. My results therefore contrast with those of our recent study on the same species, where I found that offspring survival was reduced when offspring received care from inbred parents (Mattey et al., 2013, Chapter 2). The different results between the two studies might reflect differences in experimental design. In this experiment, I assigned parents with experimental broods of 10 larvae, while Mattey et al. (2013, Chapter 2) allowed parents to raise natural brood sizes. Given that natural broods are larger on average (about 25 larvae; Smiseth and Moore, 2002) than my experimental broods, one explanation for why I failed to detect effects of inbreeding is that there were more resources available per individual offspring, and therefore less intense sibling competition. There is mounting evidence that inbreeding depression is more severe under harsh environmental conditions (Armbruster and Reed, 2005). If so, I might expect that inbreeding in parents will have a stronger effect on offspring fitness when there is more intense sibling competition.

In conclusion, this study provides the first evidence that inbreeding can moderate cooperation among male and female parents providing care for their joint offspring. Surprisingly, I found that inbreeding had a stronger effect on the amount of care provided by the partner than on the amount provided by the focal individual itself. Thus, future work on the effects of inbreeding in species with biparental care, including birds, needs to consider potential effects that are mediated through the response of the partner of inbred parents. My results suggest that the partner's response of increasing its level of care might help buffer against intergenerational effects of inbreeding in parents on the offspring's fitness. Finally, I suggest that inbreeding might have similar indirect effects on other traits that are expressed during social interactions with other

individuals. Such effects of inbreeding can be considered a form of indirect genetic effects, which occur when a trait expressed in a focal individual is influenced by the genotype of another individual with which the focal individual interacts (Mattey et al., 2013; Moore et al., 1997, Chapter 2). I suggest that inbreeding may indirectly affect other forms of cooperation, as well as other social interactions such as competition and mating, where inbreeding in one individual might impact on the behavior of individuals interacting with the focal individual, and these should be considered in future studies on inbreeding in species in which such interactions are important.

4 Effects of Inbreeding on Parent-Offspring Communication

Abstract

Inbreeding reduces an individual's survival, growth and reproduction in a wide range of taxa, but the effects of inbreeding are rarely extended to include more than one individual or to test the communication between individuals. Not accounting for the effects of inbreeding on individuals who interact with one another may underestimate the costs of inbreeding in a social group, and so the potential role of inbreeding on communication warrants further investigation. I investigated the effect of inbreeding on parent-offspring communication in the burying beetle, *Nicrophorus vespilloides*. I conducted a two-by-two factorial design where mixed broods of either inbred or outbred offspring were cross-fostered to either an inbred or outbred female parent. I recorded the subsequent mean amount of begging by individual larvae and the amount of care parents provided to each brood. My results show that inbreeding directly influenced communication between parents and offspring. Inbred offspring begged less but received more care from parents compared to outbred offspring, whilst inbred parents provided less indirect care than outbred parents. Such evidence demonstrates that it is vital to account for all the social costs of inbreeding within a family as inbreeding has strong effects on communication. It is therefore vital to incorporate social components of fitness in studies on inbreeding.

4.1 Introduction

Inbreeding is commonly associated with a reduction in fitness resulting from the expression of homozygous recessive deleterious alleles and overdominance, a phenomenon known as inbreeding depression (Charlesworth and Willis, 2009; Crow and Kimura, 1970; Falconer and Mackay, 1996; Lynch and Walsh, 1998). The fitness costs of inbreeding lead to selective pressures that shape mating systems and behaviours and pose a conservation risk to many wild populations (Armbruster and Reed, 2005; Crnokrak and Roff, 1999; Keller and Waller, 2002; Ralls et al., 1979). Therefore, it is important for both evolutionary and conservation biology to accurately estimate the fitness costs of inbreeding in a population (Charlesworth and Willis, 2009). A wide range of taxa such as birds, mammals and insects are shown to suffer reductions in traits such as survival, growth and reproduction, and such traits are commonly studied in order to accurately estimate the costs of inbreeding (DeRose and Roff, 1999; Drayton et al., 2010; Miller et al., 1993). However, the effects of inbreeding are rarely extended to include more than one individual, i.e. indirect effects of inbreeding (Fox et al., 2012; Vitikainen et al., 2011). There are no studies on the effects of inbreeding on communication, despite several studies on social behaviours such as courtship and mating success (Ilmonen et al., 2009; Okada et al., 2011). The outcome of communication, in which a signaller communicates with another individual, the receiver, is determined by both the signal and the response to it. Ignoring the effects of inbreeding on individuals who interact with one another may underestimate the costs of inbreeding in a social group (Charlesworth and Willis, 2009). Given evidence for effects of inbreeding on social behaviours such as courtship and mating (Ilmonen et al., 2009; Okada et al., 2011) and further evidence of indirect effects of inbreeding (Fox et al., 2012; Vitikainen et al., 2011), the potential role of inbreeding on communication warrants further investigation.

Individuals within a family are expected to be affected by inbreeding because they are dependent on social interactions with one another. Parent-offspring communication, i.e. the advertisement of offspring condition to parents (Kilner and Hinde, 2008), is an example of an interaction between a signaller and receiver within the family, either by affecting the offspring's signal or the parent's response. The outcome of the interactions is dynamic and complex, determined by both signaller and receiver (Royle et al., 2002b). Measuring the effects of inbreeding on communication for the both the signaller and the receiver involves investigating direct and indirect effects of inbreeding on both parents and offspring. How inbreeding affects both the signaller and receiver depends on who is in control over the provisioning of care, either parents or offspring (Parker et al., 2002). Theory offers two explanatory models: that either offspring are in control of provisioning, where offspring determine parent provisioning through

scramble competition between siblings (Macnair and Parker, 1979) or control by parents that respond to honest signals of need by offspring (Godfray, 1991, 1995). However, this is difficult to determine empirically because the two opposing models produce similar predictions and are unlikely to be mutually exclusive (Parker et al., 2002; Royle et al., 2002b). Both models predict that parents respond to the intensity of offspring's signals by providing more care, that begging is costly (Royle et al., 2002b) and that begging signals offspring need (Parker et al., 2002). The effects of inbreeding on offspring begging and parental care may provide evidence for whether parents or offspring are in control over provisioning. If offspring are in control, a key prediction is that parents passively allocate resources to the offspring, thus any alteration in offspring begging should be matched by a change in care (Parker et al., 2002). If parents are in control, a key prediction is that parents actively allocate resources, using offspring traits or begging to make decisions on who to feed (Royle et al., 2002b). Separating who responds to who is often difficult to determine because parents respond by providing more care when offspring signal, whilst offspring respond to parents providing care by increasing solicitation signals (Kilner and Johnstone, 1997). Measuring the response of the parent to both begging behaviour and the offspring's inbreeding status tests theoretical predictions on whether parents allocation is determined by the offspring's competitive strength or by the offspring's condition (Royle et al., 2002b). If inbred parents provide less care offspring will receive less care and the reduction in offspring resources may exacerbate scramble competition if offspring are in control, whilst if parents are in control fewer resources are not predicted to increase sibling competition. Thus, the difference in begging and the care provided to inbred and outbred offspring would provide information on whether begging is an honest signal or an outcome of competition, and whether parents only respond to solicitation signals.

I investigated the effect of inbreeding on parent-offspring communication in the burying beetle, *Nicrophorus vespilloides*, an excellent system for the study of both parent-offspring communication and social effects of inbreeding. Parents of *N. vespilloides* exhibit highly elaborate care and offspring exhibit tactile begging (Rauter and Moore, 1999; Smiseth and Moore, 2007; Smiseth et al., 2003). The system confirms predictions by current theory (Godfray, 1991, 1995) that offspring should beg more when hungry (Smiseth and Moore, 2004b), that parents provide more food to offspring that beg more (Rauter and Moore, 1999) and that begging is costly to offspring (Andrews and Smiseth, 2013). There are also strong direct effects of inbreeding on offspring, intergenerational effects of inbreeding in parents on outbred offspring and social effects of inbreeding on biparental cooperation (Mattey et al., 2013, Chapter 2 and 3). There is no information on how inbreeding affects offspring begging and as such my predictions of how inbred offspring beg in comparison to outbred offspring are based on evidence of both the costs associated with begging and

inbreeding effects on offspring condition (Kilner and Johnstone, 1997).

No studies have investigated the effects of inbreeding on communication and there is no information on how inbreeding affects offspring solicitation, despite a wealth of evidence for inbreeding depression in an individual's juvenile growth and survival traits (Fernandez et al., 1995; Kempenaers et al., 1996; Ralls et al., 1979) and that offspring solicitation is a vital aspect to understanding the costs imposed on inbred offspring (Godfray, 1995; Trivers, 1974). To investigate the effects of inbreeding on parent-offspring communication, I conducted a two-by-two factorial design where mixed broods of either inbred or outbred offspring were cross-fostered to either an inbred or outbred female parent. First, to investigate the effect of inbreeding on offspring communication, I compared the amount of begging by inbred and outbred offspring, and the amount of begging of offspring cared for by inbred and outbred parents and the interaction between parent and offspring inbreeding status on begging. Second, to investigate the effects of inbreeding on the response of parents to offspring communication, I compared how much care inbred and outbred parents provided, and how much care parents provided to inbred and outbred offspring and the interaction effect between parent and offspring inbreeding status on parental care. Finally, to investigate the fitness consequences of inbreeding on parent-offspring communication, I compared the growth and survival of inbred or outbred broods raised by inbred or outbred parents throughout larval development. How parent-offspring conflict and communication is resolved, and hence the amount of care that parents provide offspring, determines the growth and survival of offspring and potentially provides information on the fitness consequences of inbreeding on parent-offspring communication. Assuming that begging is costly and that inbreeding imposes physiological costs (Ayroles et al., 2009; Kristensen et al., 2006; Miller et al., 1993), I predict that inbred offspring beg less to parents. However, this does not take into account that inbred offspring will be of a reduced condition. Thus, assuming that offspring of reduced condition have increased long-term need (Godfray, 1995), I predict that inbred offspring beg more than outbred offspring, leading to parents providing more direct care to inbred offspring but not providing more indirect care. Based on the prediction that inbred offspring will beg more, and previous evidence that parents provide care to offspring that beg more (Rauter and Moore, 1999), I predict that parents will provide more care to inbred offspring that beg more. Based on previous evidence that inbred parents do not provide less care (Chapter 3), I do not predict offspring begging will differ when cared for by inbred and outbred parents, because offspring will receive the same amount of resources from both inbred and outbred parents.

4.2 Materials and Methods

4.2.1 General Methodology

All beetles used were from a large outbred laboratory population maintained at the University of Edinburgh. General husbandry procedures and the maintenance of outbreeding in the laboratory stock population were as outlined in Chapter 2. The population comprised of 14th generation beetles originally collected at Corstorphine Hill, Edinburgh; 7th and 8th generation beetles collected from Hermitage of the Braid, Edinburgh; and 7th generation beetles collected at Jodrell Bank, Manchester. Experimental pairs were bred in transparent plastic boxes (17cm x 12cm and 6cm high) by providing them with 1cm of moist soil and a previously frozen mouse carcass (supplied from Livefoods Direct Ltd, Sheffield, UK) with a mass range of 22.5 ± 2.5g. I only used virgin beetles in the experiments. Experimental outbred adults were bred using the same methods as the stock population as in Chapter 2, while experimental inbred adults derived from matings between two full siblings. Once adult, both inbred and outbred adults were maintained under standard laboratory conditions, as in Chapter 2.

4.2.2 Experimental Design

To investigate effects of inbreeding on parent-offspring communication, I used a blocked two-by-two factorial design in which mixed broods of either inbred or outbred offspring were cross-fostered to an inbred or outbred parent. This design produced a block that consisted of four treatments:(i) An outbred female caring for outbred offspring; (ii) an inbred female caring for outbred offspring; (iii) an outbred female caring for inbred offspring and (iv) an inbred female caring for inbred offspring. Outbred experimental broods were created by pooling offspring produced from experimental females mated to outbred unrelated males from the stock population. Forty-two inbred broods were created in parallel to the experimental treatments by pooling offspring produced from matings between two full siblings from the stock population. Parents of these inbred broods were not used further in the experiment as they were outbred and mated to a full-sibling, which potentially may have a confounding effect on their subsequent care (Thünken et al., 2007). I setup 20 blocks (each block containing one brood from each treatment) comprising of 80 broods, yielding data from 66: 16 broods from (i); 16 broods from (ii); 15 broods from (iii); 19 broods from (iv). Data from the original 80 broods were excluded because enough larvae could not be assigned to a treatment (n = 11) and because no larvae survived

to the time of observation (n = 3). Larvae could not be assigned to a brood in all cases because larvae die within approximately 24 hours of hatching without access to a carcass. Furthermore, because broods hatch asynchronously (Müller and Eggert, 1990) it was often not possible to create mixed broods of enough hatched larvae to allocate to a corresponding treatment. In the begging analyses, only broods in which the parent had been within distance of the offspring (the width of the parent's pronotum) during the observation period were used, as offspring only beg in the presence of a parent (Rauter and Moore, 1999; Smiseth and Moore, 2002). Based on this criteria the number of broods included in the begging analyses was reduced to 5 broods in (i); 4 broods in (ii); 10 broods in (iii) and 7 broods in (iv).

Immediately prior to the hatching of outbred eggs, the female parent and the prepared carcass were moved to a new breeding box with fresh soil, whilst the male parent was removed from the experiment. Immediately prior to inbred eggs hatching, I removed both parents and the carcass from the breeding box to allow the collection of inbred larvae. Once inbred and outbred eggs had hatched, mixed outbred broods and mixed inbred broods of ten larvae were created. Broods were standardised in this way to control for any potential effects of variation in offspring number and age on begging or parental care behaviour (Smiseth and Moore, 2002; Smiseth et al., 2003). Given that the eggs hatch asynchronously over a mean period of about 30 hours (Müller and Eggert, 1990; Smiseth et al., 2006), I continued to generate experimental broods over a period of 2-3 days. The brood mass at hatching was then recorded and the larvae were added to a corresponding inbred or outbred experimental treatment. Because parents kill any larvae that are on the carcass before their own eggs hatch, parents were only provided broods once their own eggs had hatched (Müller and Eggert, 1990; Smiseth and Moore, 2007). I carried out observations 24 hours (± 15 min) after the brood was added to the carcass, as this time period corresponds to the most active period of offspring begging and parental care in this species (Smiseth and Moore, 2007; Smiseth et al., 2003). I observed the number of offspring that were begging, parental behaviour (see below) and whether the parent was within distance of the offspring (Rauter and Moore, 1999) using instantaneous sampling each minute for 30 min (Smiseth and Moore, 2002). The mean amount of begging by individual larvae was calculated as: $b_i = (\frac{\sum b}{\sum T}/L)$ x 100 : where b = total number of begging larvae during 30 min; T = total time parent within distance of the brood; and L = brood size. I recorded parent behaviour as direct care (provisioning of food to larvae and interacting with the larvae) and indirect care (maintaining or guarding the carcass) as defined in Chapter 3. Immediately after the observation, I counted the number of larvae in the brood to record survival from hatching to observation and recorded the brood mass at observation to measure larval growth, after which the larvae were returned to the carcass. Parents were then allowed to raise the brood undisturbed until dispersal, at which point I recorded the number of larvae in order to measure survival from both

Table 4.1: Model design to test effects of inbreeding on parent-offspring communication, growth and survival of offspring. Main effects of parent and offspring inbreeding status (IS) were always included in the model whilst interaction and additional effects were included based on the lowest AIC score of all potential models. Order of the terms in each column represents the order the terms were added. Block was added as a random effect in all models

Model	Main effect	Interaction	Additional effects
Begging	Offspring IS + Parent IS	Offspring IS * Parent IS	Direct care
Parental care	Parent IS + Offspring IS	Parent IS * Offspring IS	Begging + Brood size
Growth	Offspring IS + Parent IS	Offspring IS * Parent IS	Brood size
Survival	Offspring IS + Parent IS	Offspring IS * Parent IS	Carcass size

hatching to dispersal and from observation to dispersal and I also recorded brood mass at dispersal.

4.2.3 Statistical Analysis

Data were analysed using R version 2.13. Linear mixed-effects models were used for traits that had a normal error distribution (offspring growth) and generalised linear mixed models were used for traits with a Poisson error structure (offspring begging and parental care) or a binomial error structure (offspring survival). All final models included both the main effects of parent and offspring inbreeding status, while the inclusion of the interaction and additional effects was based on the lowest AIC score. If a difference in the AIC score was less than two then the simplest model was chosen (Table 4.1). Block was assigned as a random factor in all models. I built separate mixed effects models for offspring begging, parental care, offspring growth and offspring survival, and all models comprised of three parts: main effects, an interaction, and additional effects (Table 4.1). Begging models were analysed using the mean proportion of larvae begging as the response variable. I built mixed effects models with the time that female parents spent providing direct care as the response variable and separate models with indirect care as the response variable. In parental care models, brood size was included as an additional effect based on AIC score and female parents were strongly affected by the size of the brood (direct care: $Z = 3.86$, $P = 0.0001$; indirect care: $Z = 3.39$, $P = 0.0007$). I built separate mixed effects models for offspring growth and offspring survival. I included brood size as an additional effect in the offspring growth models based on AIC score and larvae were significantly bigger in larger broods at observation and dispersal (Table 4.2). I included carcass size as an additional effect in offspring survival models based on AIC score and offspring had reduced survival on larger mouse carcasses from hatching to observation and hatching to dispersal (Table 4.2).

To examine whether any potential effects of inbreeding in parents on offspring begging were independent of the amount of parental care I included the amount of direct care parents provided as an additional fixed effect in the begging analyses (Table 4.2). I then compared the effect of inbred parents on offspring begging when this additional fixed effect was excluded or included in the model. This was repeated for the parental care models to again test whether the effects of inbred offspring on parental care were independent of offspring begging. Here, I compared the effect of inbred offspring on parental care when the additional fixed effect of offspring begging was excluded or included in the model.

4.3 Results

4.3.1 Offspring Begging

Inbred offspring begged significantly less than outbred offspring (Figure 4.1; Table 4.2). Offspring begged more to inbred parents, though this latter effect was considerably weaker than that of the offspring's own inbreeding status (Figure 4.1; Table 4.2). There was no evidence of an interaction between offspring and parent inbreeding status on offspring begging (Table 4.2). When excluding direct care from the begging model, the parent's inbreeding status remained significant ($Z = 3.16$, $P = 0.002$). This result suggests that the effect of inbreeding in parents on offspring begging was independent of the amount of care provided by parents.

4.3.2 Parental Care

Inbred parents provided significantly less indirect care than outbred parents whilst inbred and outbred parents did not differ in the amount of direct care they provided (Figure 4.2; Table 4.2). Parents provided more direct and indirect care to inbred offspring compared to outbred offspring, though for direct care this only occurred when parents themselves were also outbred. This was evidenced by a statistical interaction between offspring and parent inbreeding status (Figure 4.2; Table 4.2). Outbred parents provided more direct care to inbred offspring than to outbred offspring, whilst inbred parents did not differ in the amount of direct care provided to inbred and outbred offspring (Figure 4.2; Table 4.2).

Including or excluding offspring begging did not change the effect that the offspring's inbreeding status had on the amount of care provided by parents: parents of inbred

offspring still spent significantly more time providing direct care to inbred offspring when excluding offspring begging from the direct care model (Z = 4.64, $P < 0.0001$). Excluding offspring begging from the parental care model altered the effect of the offspring's inbreeding status on the parent's indirect care. As such, when offspring begging was excluded from the indirect care model parents of inbred and outbred offspring did not differ in the amount of direct care they provided (Z = 1.6, $P = 0.11$; compared with when begging was included: Table 4.2). These results therefore suggest that the effect of inbred offspring on the amount of direct care provided was independent of offspring begging, whilst the effect of inbred offspring on the amount of indirect care provided was not independent of offspring begging.

4.3.3 Growth and Survival of Offspring

Inbred offspring were smaller than outbred offspring when they were cared for by outbred parents, whilst there was no difference in the size of inbred and outbred offspring when they were cared for by inbred parents (Figure 4.3 a and b; Table 4.3). The survival of inbred offspring was lower than that of outbred offspring during the parental care period, from observation to dispersal and from hatching to dispersal (Figure 4.3 c and d; Table 4.3). In addition, the survival of offspring raised by outbred parents was also lower than the survival of offspring raised by inbred parents at the dispersal stage of larval development, from observation to dispersal and from hatching to dispersal (Figure 4.3c and d; Table 4.4). However, this latter effect of a reduction in survival of offspring cared for by outbred parents only occurred when offspring themselves were inbred, as evidenced by an interaction between parent and offspring inbreeding status (Figure 4.3c and d; Table 4.4).

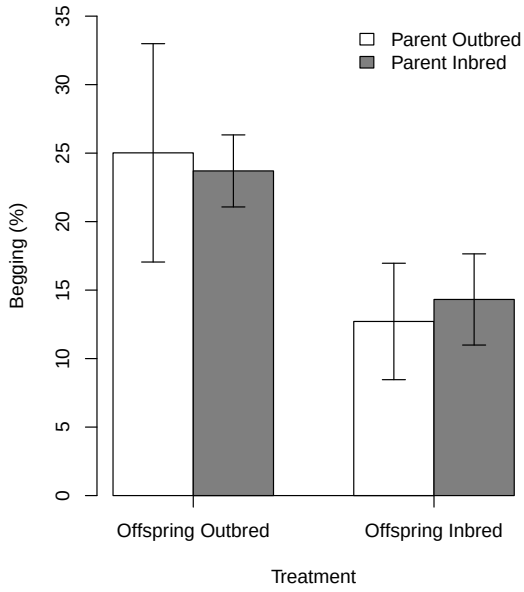

Figure 4.1: Effects of inbreeding in *Nicrophorus vespilloides* on the % of time larvae begged to a female parent during a 30min observation. Comparisons between outbred (white bars) and inbred parents (grey bars) caring for by outbred or inbred offspring (Treatment) (mean ± SE).

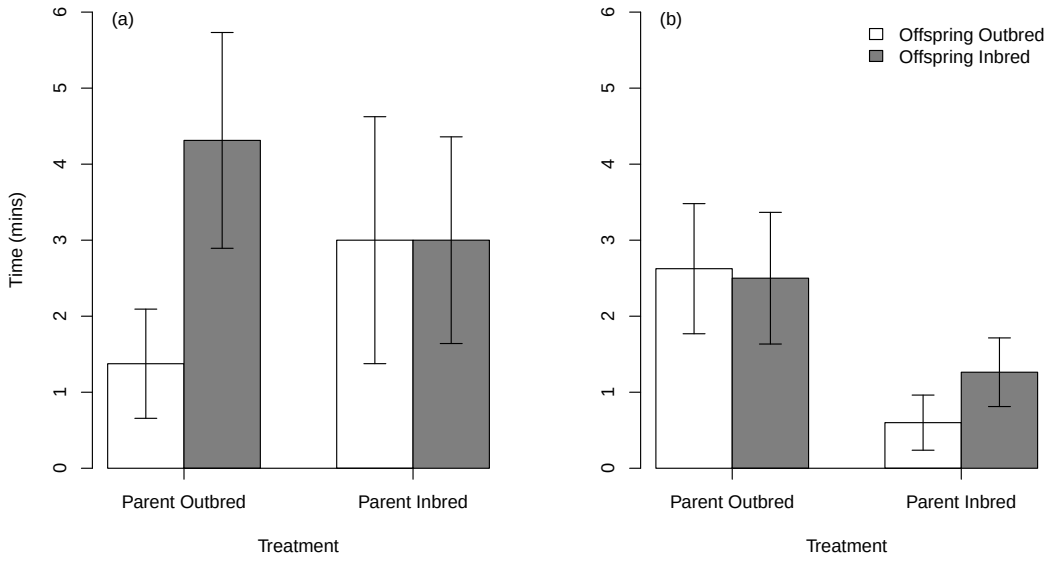

Figure 4.2: Effects of inbreeding in *Nicrophorus vespilloides* on the amount of time female parents provided direct care (a) and indirect care (b). Comparisons between outbred (white bars) and inbred (grey bars) offspring cared for by outbred or inbred female parents (Treatment) (mean ± SE).

Table 4.2: Effects of inbreeding on parent-offspring communication in *Nicrophorus vespilloides*. Parameter estimates (Par), standard error (SE), Z-value and P- value for the effects for the offspring's inbreeding status (IS), the parents IS, their interaction (P*O) and the behaviour of the interacting individual (i.e. the amount of direct care provided by parents was included for begging analyses whilst offspring begging % was included in direct and indirect care analyses).

	Offspring				Parent				P*O				Behaviour			
Trait	Par	SE	Z-Value	P-Value	Par	SE	Z-Value	P-Value	Par	SE	Z-Value	P-Value	Par	SE	Z-Value	P-Value
Begging	-0.64	0.12	-5.34	<0.0001	0.37	0.18	2.05	0.041	1.12	0.25	0.49	0.63	0.022	0.01	2.06	0.039
Direct care	1.26	0.22	5.84	<0.0001	-0.17	0.22	-0.76	0.44	-1.38	0.41	-3.37	0.0007	0.11	0.01	9.92	<0.0001
Indirect care	0.48	0.21	2.31	0.021	-0.92	0.23	-4.06	<0.0001	0.9	0.47	1.92	0.055	0.035	0.01	4.09	<0.0001

Table 4.3: Effects of inbreeding on offspring growth in *Nicrophorus vespilloides*. Parameter estimates (Par), standard error (SE), t-value and P-values for the effects for the offspring's inbreeding status (IS), the parents IS, their interaction (P*O) and the effect of brood size.

	Offspring				Parent				P*O				Brood size			
Trait	Par	SE	t-Value	P-Value	Par	SE	t-Value	P-Value	Par	SE	t-Value	P-Value	Par	SE	t-Value	P-Value
Hatching	-3.6×10^{-5}	5.6×10^{-5}	-0.46	0.65	-7×10^{-6}	5.6×10^{-5}	-1.21	0.23	-6×10^{-5}	1.1×10^{-4}	-0.56	0.58	-	-	-	-
Observation	-0.001	0.0007	-1.36	0.18	-0.0012	0.0008	-1.56	0.13	0.0012	0.0002	6.85	<0.0001	0.0012	0.0002	6.94	<0.0001
Dispersal	-0.0007	0.0053	0.13	0.9	-0.0024	0.0054	-0.44	0.66	0.0068	0.0011	5.95	<0.0001	0.0069	0.0011	6.05	<0.0001

Table 4.4: Effects of inbreeding on offspring survival in *Nicrophorus vespilloides*. Parameter estimates (Par), standard error (SE), t-value and P-values for the effects for the offspring's inbreeding status (IS), the parents IS, their interaction (P*O) and the effect of carcass size.

	Offspring				Parent				P*O				Carcass Size			
Trait	Par	SE	Z-Value	P-Value	Par	SE	Z-Value	P-Value	Par	SE	Z-Value	P-Value	Par	SE	Z-Value	P-Value
Hatching to observation	-0.12	0.22	-0.54	0.59	-0.36	0.23	-1.55	0.12	0.83	0.45	1.86	0.062	-0.37	0.14	-2.72	0.0066
Observation to dispersal	1.6	0.21	7.72	<0.0001	-0.96	0.24	-3.98	<0.0001	0.88	0.49	1.82	0.069	-0.16	0.12	-1.35	0.18
Hatching to dispersal	0.41	0.18	2.28	0.023	-0.7	0.19	-3.74	0.0002	1.14	0.37	3.11	0.0019	-0.35	0.11	-3.25	0.0011

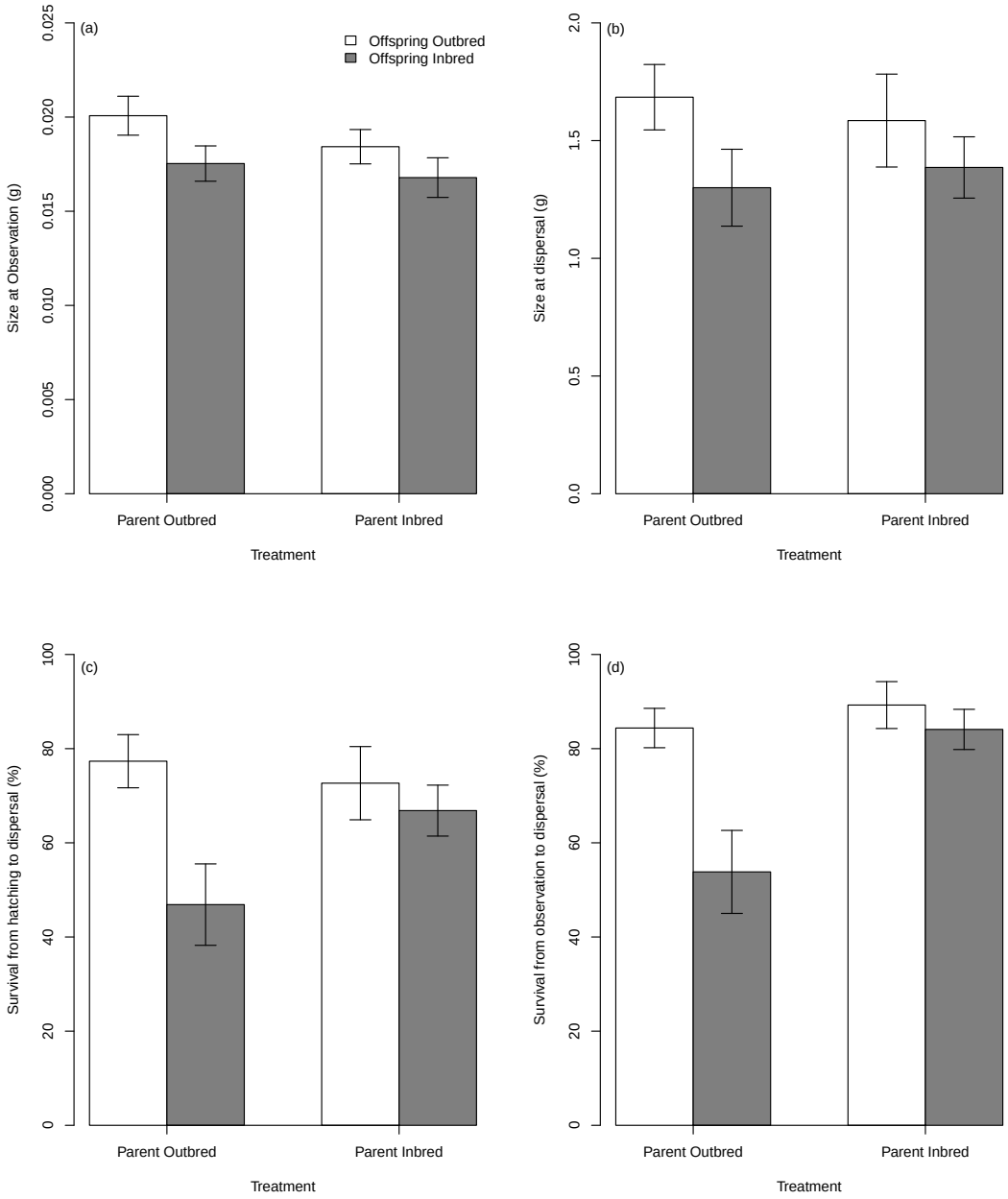

Figure 4.3: Effects of inbreeding on the growth (a - b) and survival (c - d) in *Nicrophorus vespilloides*: this is shown 24 hours after hatching (a and c) and at dispersal (b and d). Comparisons between outbred (white bars) and inbred (grey bars) offspring cared for by an outbred or inbred female parent (Treatment) (mean ± SE).

4.4 Discussion

I show for the first time that inbreeding directly influenced communication between parents and offspring, an effect that is expected to affect the fitness of all individuals within the family. Inbreeding in both parents and offspring affected offspring begging

independently, but parent and offspring inbreeding status also interacted with one another, affecting the amount of direct care provided by parents. The statistical interaction suggests that the inbreeding status of both signaller and receiver influences the outcome of parent-offspring communication. Inbred offspring begged less but received more care from parents compared to outbred offspring, whilst inbred parents provided less indirect care than outbred parents. As well as begging less and receiving more care, inbred broods raised by outbred parents grew and survived less well during the parental care period. A statistical interaction between parent and offspring inbreeding status suggests that the growth and survival of offspring depended on both the inbreeding status of the signaller and of the receiver, influenced by the communication between parents and offspring. While providing evidence that it is vital to account for all the social costs of inbreeding within a family, these results also raise questions on additional cues to begging from offspring, inbreeding interactions with the social environment and whether inbreeding affects other aspects of parental care. Below I discuss the wider implications of these results in more detail.

Inbred offspring begged less than outbred offspring and the offspring's inbreeding status was shown to have a more significant effect than the parent's. That inbred offspring begged less than outbred offspring is in contrast to the prediction that inbred offspring would beg more. This result is surprising given that inbred offspring survive less well than outbred offspring (Mattey et al., 2013), that there is no evidence of energetic costs of begging in *N. vespilloides* (Smiseth and Parker, 2008) and that parents provided more direct care to inbred offspring (Table 4.2). I suggest that the lower levels of begging by inbred offspring was caused by the effect of inbreeding on the condition of the offspring and the costs of begging. As inbred offspring begged less than outbred offspring, the results suggest that they suffered behavioural or energetic costs to inbreeding. These costs reduced offspring's ability to solicit food from parents, and any increase of need to solicit food did not outweigh these behavioural or physiological constraints of inbreeding. The reduction in begging by inbred offspring cannot be explained as an effect of the amount of care provided. First, if provisioning by the parent explained the begging by offspring it would be predicted that the pattern of begging and direct care would reflect one another, however this is not the case when comparing Figure 4.1 and 4.2 a. In particular, the interaction term between offspring and parent inbreeding status present for direct care was not present for begging. The direct care inbred parents provided did not differ between inbred and outbred offspring, but inbred offspring still begged less than outbred offspring when cared for by an inbred parent. Therefore the patterns of begging are different from those for the parent's provisioning. Second, whilst offspring begging was affected by the amount of direct care parents provided, I show that offspring begged more, rather than less, to parents who provided more direct care (Table 4.2). However, the effect of inbred parents on offspring begging was weak (Table 4.2), and given the small sample

size for begging the offspring's begging response to an inbred parent needs to be tested further. Altogether, these results suggest that the physiological costs imposed by inbreeding outweighed the need for inbred offspring to solicit more care from parents, leading to inbred offspring begging less than outbred offspring, independent to the care provided by parents.

Inbred offspring not only begged less but indirectly affected the care parents provided. I show evidence that parents were responsive to the inbreeding status of their offspring, providing more direct and indirect care to inbred offspring. This higher amount of care provided to inbred offspring is unlikely to be due to variation in offspring begging. First, outbred offspring begged more than inbred offspring and so offspring who begged more were not obtaining more care from the parent. Second, the amount of begging by inbred offspring did not explain the variation in the amount of direct care provided by parents, and removing the term from the parental care model did not change the effect that parents provided more care to inbred offspring. Therefore, given that parents provided more care to inbred offspring and that inbred offspring begged less than outbred offspring, I suggest that parents used cues other than begging to determine the amount of care to provide inbred or outbred offspring. Predictions by honest signalling models assume that parents are unable to assess offspring condition directly, relying on offspring begging as a signal of their need (Godfray, 1991, 1995). My results suggest that parents are using another signal. Potential cues include volatile chemical cues acting as a signal from the brood as a whole or cuticular hydrocarbons (CHCs) acting as signals from individual offspring, these cues are discussed further below. In addition, an interaction between parent and offspring inbreeding status showed that only outbred parents provided more direct care to inbred offspring, with no such difference in the amount of care provided by inbred parents to inbred or outbred offspring. This interaction suggests that inbred parents could not adjust to the inbreeding status of their offspring, as shown in Chapter 3, and that inbred female parents were less responsive to the inbreeding status of the individual they interacted with.

The effect of inbreeding on parent-offspring communication suggests that provisioning is under the parent's control. The nature of parent-offspring communication means that the provisioning and begging behaviours are particularly difficult to interpret independently as each strongly influences the other (Godfray, 1991, 1995). However, I present evidence that supports the theory that parents were in control over the amount of care provided to offspring. Evidence supports that parents control provisioning by allocating care based on cues in addition to begging, as discussed above. I show that parents provide more direct care to inbred offspring who beg less and survive less well, and so appear to assess the need of offspring independently to begging and do not provision to the strongest offspring signals, a key prediction to honest signalling

models (Royle et al., 2002b). The potential mechanism of an alternative cue based on offspring need are unlikely to be under offspring control as such signals do not meet scramble competition model predictions. The cues may be volatile chemical cues from the brood as a whole, or CHCs from individual offspring. I would argue that neither of these cues give offspring the potential to control provisioning based on competition. A volatile chemical cue may act as a signal from the whole brood, but do not allow the potential for use in intra-brood competition, as assumed in offspring control models (Macnair and Parker, 1979). Cuticular hydrocarbons would allow individual offspring the opportunity to compete with siblings over the provision of care, though to signal to the parent would require contact. Cuticular hydrocarbons cues would therefore likely act through contact with the parent, which in this study would be recorded as begging behaviour. In addition, offspring control models predict that the allocation of provisioning using begging signals would lead to parents providing less care to inbred offspring, as offspring begged less than outbred offspring. Furthermore, the interaction on the direct care provided suggests that parents response was based on both their offspring's inbreeding status and their own inbreeding status. Therefore, the response of parents to inbred offspring suggest that at this stage, parents were likely to be in control over the allocation of care.

The amount of care parents provided was also dependent on their own inbreeding status. Contrary to predictions and previous results (Chapter 3), I found that inbred parents provided less care than outbred parents (Table 4.2). I show that inbred parents provided less indirect care (though not in Chapter 3), and I suggest that this may be explained by the difference in experimental design, which differed in whether offspring were raised in the absence or presence of the male parent. The difference in providing care in the presence or absence of a partner may explain the reduction in care by female parents in a different social environment. The lower amount of indirect care provided by inbred parents in the absence of a partner suggests that parents caring without a partner suffered higher inbreeding costs to those caring with a partner. Parents may suffer higher costs in such a situation because inbreeding interacted with the social environment, that is whether parents provide care in the presence or absence of a partner. A study on a wild population of meerkats, *Suricata suricatta*, suggests that the social environment 'buffers' the severity of inbreeding depression, because inbreeding depression was only present at a stage when helpers discontinue provisioning, leading to a harsher social environment for inbred offspring (Nielsen et al., 2012). Furthermore, inbreeding depression depends on the presence or absence of the maternal parent in the European earwig, *Forficula auricularia*, providing more evidence that a more stressful social environment may increase the effects of inbreeding (Meunier and Kölliker, 2013). My results suggest that a harsher social environment was created by the absence of a male parent and lead to increased costs of inbreeding for the female parent, with the female providing less indirect care.

This is in contrast to previous evidence which suggests that widowed females suffer no costs (Smiseth et al., 2005). While there is growing interest in the study of the environmental interaction effects of inbreeding, I suggest that these studies should be extended to investigate whether the social environment interacts with inbreeding.

The effects of inbreeding on the growth and survival of offspring in relation to the effects of inbreeding on parent-offspring communication were difficult to interpret using the behavioural results, as whilst inbred offspring begged less (indicating a worse condition), they received more direct care from parents. Although the growth and survival of offspring to some degree matched the patterns of parental care that offspring received, the variation in both growth and survival were not explained by the care that parents provided. As I found for the amount of direct care provided, there was no difference in the growth and survival of offspring cared for by inbred parents, but there was a difference between inbred and outbred offspring cared for by outbred parents (Figure 4.2 a; 4.3). However, unlike the amount of direct care provided, inbred offspring grew and survived less well than outbred offspring, rather than surviving better, as predicted by the finding that inbred offspring received more direct care (Figure 4.2 a; 4.3). The reduction in growth and survival of offspring therefore suggests that inbred offspring were of a poorer physical condition. Conversely, the interaction between parent and offspring inbreeding status shows that inbred offspring only survived less well when cared for by outbred parents, and that inbred and outbred offspring cared for by inbred parents did not differ in survival to outbred offspring cared for by outbred parents (Figure 4.3 a and b). The statistical interaction is surprising, because inbred offspring did not differ in begging behaviour when raised by inbred or outbred parents, but inbred and outbred parents did differ in the amount of care provided to inbred offspring (Figure 4.1; 4.2 a). The statistical interaction is also in contrast to the survival of inbred offspring cared for by inbred parents, as inbred offspring received more direct care than outbred offspring cared for by inbred parents (Figure 4.1; 4.2 a). Therefore, inbred offspring cared for by inbred parents would be expected to grow and survive less well than inbred offspring cared for by outbred parents, as they suffered the same costs of inbreeding in themselves, but received less care from parents. This contrast between inbred offspring cared for by inbred and outbred parents suggests that, as in Chapter 3, there appears to be a benefit in terms of survival to offspring that were raised by an inbred parent, that is unexplained by the amount of care provided. Other parental factors that impact offspring growth and survival may explain the effects of inbreeding on offspring growth, such as the quality of care, carcass preparation and social immunity provided by parents (Arce et al., 2012; Cotter and Kilner, 2010b; Rozen et al., 2008). The effects of inbreeding on such factors should be investigated to establish the mechanisms by which inbred and outbred parents influence offspring fitness.

In conclusion, the effects of inbreeding on parent-offspring communication, growth and survival were mediated through parents who allocate care to offspring based on cues other than begging. However, social effects of inbreeding may mediate such control, and therefore the growth, survival and care offspring receive depend on whether the parent is inbred or outbred. Inbreeding has strong effects on communication for both the signaller and receiver. In this context, inbreeding affects intra-familial conflict and potentially parent-offspring communication. It is therefore vital to incorporate social components of fitness in studies on inbreeding.

5 Effects of Inbreeding on The Social Immunity of Parents

Abstract

There is growing evidence for intergenerational effects of inbreeding, i.e the reduction in fitness of outbred offspring produced by inbred parents, and the mechanisms are as yet undetermined. In many species the immune protection parents provide has strong effects on offspring fitness, but whether inbreeding affects the social immune function of parents has not previously been investigated. *Nicrophorus vespilloides* offspring are raised on a small vertebrate carcass, in which parents provide care by applying anal and oral secretions that protect the carcass and offspring from microbial competitors and pathogens. I investigated the effects of inbreeding on the social immunity of parents by creating four independent treatments in which focal parents were either male or female and either inbred or outbred. I collected secretions from focal parents during three distinct stages of offspring development and tested the bactericidal and lytic activity of the secretions. I found the first example of inbreeding effects on the social immunity of parents. I show that the social immunity of male parents was affected by inbreeding, reducing the bactericidal activity at the latter stage of offspring development. However, this effect of inbreeding depended on both the sex of the parent and the stage of offspring development and the social immune functions of inbred parents remains high during the most important stages of offspring development.

5.1 Introduction

Inbred individuals suffer a reduction in fitness (Crow and Kimura, 1970; Falconer and Mackay, 1996; Lynch and Walsh, 1998), and there is growing evidence that suggests indirect effects of inbreeding, i.e the effect of inbreeding in one individual that also affects the fitness of another individual, may lead to further costs that influences the fitness of other individuals in a social group, such as a family (Fox et al., 2012; Mattey et al., 2013; Reid et al., 2003; Richardson et al., 2004; Szulkin et al., 2007, Chapter 2). How inbreeding indirectly affects other individuals within a social group is likely to depend on the context and relationship between two individuals and how particular social traits affect each individual's fitness. For example, the reduction in hatching success of eggs laid by outbred females mated to inbred males may be due to inbred males having reduced sperm quality or mating ability (Fox et al., 2012). In contrast, intergenerational effects of inbreeding, i.e. the reduction in fitness of outbred offspring raised by inbred parents, may depend on the quantity or quality of care provided by inbred parents (Mattey et al., 2013; Reid et al., 2003; Szulkin et al., 2007, Chapter 2). The mechanisms for intergenerational effects of inbreeding within a family have the potential to be particularly complex as offspring fitness depends on a suite of parental traits such as provisioning care and protecting or maintaining environment offspring are raised in (Clutton-Brock, 1991). The causes of intergenerational effects may provide information on the importance of particular parental traits and how such parental traits affect offspring fitness.

Parental care is suggested as a mechanism responsible for the reduction in fitness in outbred offspring cared for by inbred female parents in several species (Mattey et al., 2013; Reid et al., 2003; Szulkin et al., 2007, Chapter 2). In dairy cattle, inbred parents have increased somatic cell counts, a trait linked to a reduction in health and milk production due to its role in protecting maternal parents from mastitis (Mc Parland et al., 2007; Miglior et al., 1995). However, there is no evidence that inbreeding reduces the amount of care provided by parents such that it leads to intergenerational effects on outbred offspring in non-domestic animals (Margulis, 1998; Richardson et al., 2004, Chapter 3). However, while the amount of parental care provided has been investigated, the quality of care provided by inbred parents has not been investigated (Margulis, 1998, Chapter 3). For example, the amount or quality of food provided to offspring, or the immune protection provided to offspring may be reduced in inbred parents. Evidence supports that the amount of time spent providing care is a good measure of care, which shows that the amount of provisioning provided by parents is strongly associated with offspring fitness (Eggert and Müller, 1997; Smiseth et al., 2003). There is evidence that both the amount of immune protection parents provide and how good the parent's social immunity is at providing protection to

offspring have strong effects on offspring fitness (Cardoza et al., 2006; Fleming et al., 2009; Giacomello et al., 2006; Rozen et al., 2008). Inbreeding often has strong effects on the personal immune response of individuals (Reid et al., 2007; Townsend et al., 2010), though evidence in invertebrates is mixed. For example, inbred autumnal moths, *Epirrita autumnata*, and bumble bees, *Bombi terrestris*, suffer reductions in their cellular immune response and life-history traits (Rantala and Roff, 2007; Whitehorn et al., 2011), while inbred crickets, *Gryllodes sigillatus*, *Teleogryllus commodus* and wood ants, *Formica exsecta* have increased immune function (Drayton and Jennions, 2011; Gershman et al., 2010; Vitikainen et al., 2011). Currently, there is no evidence on the effects of inbreeding on the social immune function of parents. I use the broad definition of social immunity as an immune response by one individual that increases the fitness of the actor and recipient (Cotter and Kilner, 2010a). In contrast, social immunity by parents towards offspring is found in several species, whereby parents secrete substances that protect eggs or offspring from microbial infections or competition (Cardoza et al., 2006; Cotter and Kilner, 2010a; Fleming et al., 2009; Giacomello et al., 2006), but the effects of inbreeding on social immunity are unknown. Thus, the protection parents provide offspring against pathogens and microbial competitors is a form of parental care that gas not yet been investigated as a possible mechanism for intergenerational effects of inbreeding.

Nicrophorus vespilloides offspring are raised on a small vertebrate carcass, in which parents feed pre-digested carrion to offspring, and from which offspring can also self-feed (Eggert et al., 1998; Scott, 1998). Parents also provide care by applying anal and oral secretions that protect the carcass and offspring from microbial competitors and pathogens (Eggert et al., 1998; Rozen et al., 2008). The secretions that parents apply to the carcass mean that *N. vespilloides* are an excellent study system to test the effects of inbreeding on social immunity, as they also suffer substantial direct, intergenerational and social effects of inbreeding (Mattey et al., 2013, Chapter 2, 3, 4). In particular, outbred offspring survive less well when raised by inbred female parents, though only when male parents are outbred (Mattey et al., 2013, Chapter 2). Whilst inbreeding in *N. vespilloides* has complex effects on the amount of care male and female parents provide, the care parents provide does not explain the intergenerational effects of inbreeding on the survival of outbred offspring (Mattey et al., 2013, Chapter 2, 3). However, inbred parents may affect offspring fitness by other mechanisms, as the survival of offspring depends on both the amount of time spent providing care, and how well the carcass is protected from competitors (Arce et al., 2012; Rozen et al., 2008). A number of studies have investigated personal immunity, social immunity and the trade-off between the two in *Nicrophorus*. The activity of the secretions appear to be costly and have both bactericidal and lytic effects due to a beetle lysozyme that appears to be upregulated during offspring development (Arce et al., 2012; Cotter and Kilner, 2010b; Cotter et al., 2010a, 2013; Steiger et al., 2011). Both the lytic and

bactericidal activity of the secretions is higher in female parents than in male parents, and females have particularly high activity at the early stages of larval development (Cotter and Kilner, 2010b; Steiger et al., 2011). The activity of the secretions varies throughout offspring development, peaking at larval hatching before decreasing again at dispersal (Arce et al., 2012; Cotter and Kilner, 2010b). The composition of anal secretions is highly diverse and it has been hypothesised that such components might vary with physiological state of parents (Degenkolb et al., 2011). Hence, a reduction in fitness as a result in inbreeding may lead to further variation in the activity of parent's secretions.

I investigated the effects of inbreeding on the social immunity of male and female parents in *N. vespilloides*. I created four independent treatments in which focal parents were either male or female and either inbred or outbred, mated to an outbred unrelated partner. I collected secretions from focal parents during three distinct stages of offspring development. As outbred offspring survived less well when raised by inbred female parents (Mattey et al., 2013, Chapter 2), I predict that inbred female and male parents' social immunity provides less protection against bacteria than outbred parents. Given that female parents have higher social immune function during larval development than males (Cotter and Kilner, 2010b), I predict that female parent's social immunity would be more affected by inbreeding than that of the male's. Because lytic and bactericidal activity changes throughout larval development, I also predicted that inbreeding effects would be greatest at the point in which the secretions of parents have highest activity, at the hatching stage when larvae first arrive at the carcass which is the most significant stage for larval survival (Arce et al., 2012; Cotter and Kilner, 2010b).

5.2 Materials and Methods

5.2.1 General Methodology

All beetles used were from a large outbred laboratory population maintained at the University of Edinburgh. General husbandry procedures and the maintenance of outbreeding in the laboratory stock population were as outlined in Chapter 2. All beetles in the experiment comprised 3rd to 7th generation beetles originally collected at Corstorphine Hill, Edinburgh and 11th and 12th generation beetles collected from Hermitage of the Braid, Edinburgh. Experimental pairs were bred in transparent plastic boxes (17cm x 12cm and 6cm high) by providing them with 1cm of moist soil and a previously frozen mouse carcass (supplied from Livefoods Direct Ltd, Sheffield,

UK) with a mass range of 22.5 ± 2.5g. All beetles used were virgin. Experimental outbred adults were bred using the same methods as for the stock population (Chapter 2), while experimental inbred adults derived from matings between two full siblings. Once adult, both inbred and outbred adults were maintained under standard laboratory conditions, as in Chapter 2.

5.2.2 Experimental Design and Secretion Collection

The experimental design comprised of four independent treatments with one focal parent mated to an outbred unrelated partner. The four treatments contained one focal parent from whom all secretions were collected, this focal parent was either an: (i) outbred female; (ii) inbred female; (iii) outbred male; (iv) inbred male. The partner of the focal parent remained in the breeding box until dispersal, to measure inbreeding effects on social immune function in a biparental system. I set-up 120 broods, comprising of 30 broods per treatment. Secretions were collected at three stages from the same focal parent: when eggs were first observed (egg); when larvae were first observed hatching (hatching); and when larvae had dispersed from the carcass (dispersal). At each stage, secretions were collected from the focal parent by isolating the liquid secretions from disturbed beetles when lifted with forceps, as in Arce et al. (2012). I recorded the volume of secretion collected and it was subsequently diluted in 9µl sterile phosphate-buffered saline (PBS), flash frozen on dry ice and stored at -80°C. Each sample was then diluted to 10% (vol/vol) in PBS. Eleven broods were excluded from the data set because one parent died (n=9) or because the female laid no eggs (n=2). Larvae did not hatch from any eggs in 28 broods and so these broods were excluded from the analyses on hatching and dispersal. In some instances beetles secreted zero, or less than 10µl, volume of secretions, and 51 samples were therefore not collected and subsequently no included in the analyses. To test both the lytic and bactericidal activity of secretions 14µl was required, however only 10µl was collected in 78 broods. Samples with less than 14µl of diluted secretions were randomly split in half at each stage and assigned to either the bactericidal or lytic assay. Sample sizes are provided for each analysis in the Results section.

5.2.3 Lytic Activity

The degradation rate of *Micrococcus lysodeikus* bacterial cell walls by the secretions from the parents was measured using a microplate assay based on Arce et al. (2012). Ten µl of parent's secretions or control sample of PBS and 90µl of 3mg/ml suspension of bacterial cell walls in ultrapure water was added to a 96-well microtitre plate.

Plates were then incubated at 37°C with continuous shaking in an automated plate reader (BioTek EL808) that measured the optical density (OD) at 600nm every 15s for 10 min. Lytic activity was estimated by calculating the proportional change in OD over 10 min between experimental and control samples (Arce et al., 2012).

5.2.4 Bactericidal Assay

The bactericidal activity measured the decline in live bacterial *Bacillus subtillis* cells from an overnight incubation after the addition of dilute secretions of parent's secretions or control sample of PBS, based on the protocol used by Arce et al. (2013). All samples were first centrifuged at 16000g for 2 min in order to remove debris. 1ml of 10^6 *B. subtillis* cells/ml were added to 25ml lysogeny broth (LB) agar and plated, on which 4 µl of secretions or control PBS were added, and incubated overnight at 37°C. The presence of bactericidal activity was then recorded by the presence of clearance zones and for samples with zones present the diameter was measured to test the strength of the bactericidal activity.

5.2.5 Statistical Analysis

Data were analysed using R version 2.13. Linear mixed effects models were used for traits that had a normal error distribution (lytic activity and the strength of bactericidal activity) and generalised linear mixed models were used for traits with a binomial error structure (presence of bactericidal activity). Samples were repeated measurements and so non-independent. To account for this, the focal parent ID was added as a random factor in all models. All final models were based on the lowest AIC score, if a difference in the AIC score was less than two then the simplest model was chosen. To investigate the lytic activity of the parent's secretions, I tested whether the proportional decline in OD after 10 min relative to the control was affected by inbreeding status (outbred or inbred), sex (male or female) and stage of collection (egg, hatching or dispersal). Models containing these fixed effects and all possible interactions between the terms were included and the model with the lowest AIC score was chosen. The final lytic activity model did not include any interaction terms between the fixed effects, based on AIC scores.

To analyse the bactericidal activity of parent's secretions on *B. subtillis* I tested whether the presence of bactericidal activity was affected by inbreeding treatment, sex, stage and the presence of bacteria in the parent's secretions (present or absent). This latter fixed effect was fitted to account for the effect that some secretion samples

Table 5.1: Effects of inbreeding on breeding male and female lytic activity in *Nicrophorus vespilloides* secretions during egg laying, hatching and dispersal. For each factor parameter estimates (Par) with standard error (SE), degrees of freedom, t-values and *P*- values compare outbred and inbred male or female parents and stage. n = 215.

Factor	Par	SE	Degrees of Freedom	*t*-Value	*P*-Value
Treatment	0.0188	0.14	112	0.13	0.897
Sex	0.421	0.14	112	2.9	0.004
Stage (hatching)	-1.11	0.17	98	-6.48	<0.0001
Stage (dispersal)	-0.454	0.17	98	-2.72	0.008

grew an a contaminant bacteria during overnight incubation, likely to have been present in the secretions. I tested models in which all possible interactions between inbreeding treatment, sex and stage were included. The final model included a three-way-interaction between inbreeding status, sex and stage and a main effect of the presence of bacteria in the parent's secretions. To interpret this three-way-interaction I carried out two separate analyses on females and males. Models were created and chosen with fixed effects as in the previous analysis but without sex as a fixed effect. Based on AIC score, both female and male models contained an interaction term between inbreeding treatment and stage and did not contain the additional fixed effect of the presence of bacteria in the parent's secretions. In order to test the strength of bactericidal activity (zone of clearance diameter) of the secretions, I removed all zero values in a separate analysis. I fitted the inbreeding status, sex, stage, all possible interactions between these terms, and whether bacteria was present in the sample after incubation. The final model of bactericidal strength did not include any interaction terms or the effect of the presence of bacteria in the parent's secretions.

5.3 Results

5.3.1 Lytic Activity

The lytic activity of inbred and outbred male and female parents did not differ during any stage of offspring development (Figure 5.1; Table 5.1). However, male parents had higher lytic activity than females during the egg laying ‘ and dispersal stages, whilst there were no differences between males and females during the hatching stage (Figure 5.1 a-c; Table 5.1). The lytic activity did change significantly over the egg, hatching and dispersal stages, as activity was lower during the hatching and dispersal stages than at the egg stage (Figure 5.1 a-c; Table 5.1).

5.3.2 Bactericidal Activity

The presence of bactericidal activity was not influenced by the main effects of inbreeding status and sex, though it did vary depending on the stage of offspring development. The presence of activity increased from the egg to the hatching stage, whilst the presence of activity was significantly lower at the dispersal stage than at the hatching or egg stage (Figure 5.1 d-f; Table 5.2). Females and males differed in the presence of bactericidal activity depending on stage, as the presence of activity was higher in males at dispersal, compared to the egg stage (Figure 5.1 d-f; Table 5.2). A three-way-interaction between inbreeding status, sex and stage was present during dispersal, and to interpret this interaction further I split the analysis into separate male and female analyses (Table5.3). The presence of activity in female parents was significantly lower at the dispersal stage than at the hatching or egg stage but were not affected by inbreeding (Table 5.3: see female data). Male parents had higher presence of activity at hatching, which then decreased again at the dispersal stage. However, inbred male parents had a lower presence of activity than outbred male parents at dispersal, whilst inbred and outbred males did not differ at the egg and hatching stages (Figure 5.1 d-f; Table 5.3). The strength of bactericidal activity did not differ significantly between inbred and outbred parents, male and female parents or between different stages of larval development (Table 5.4).

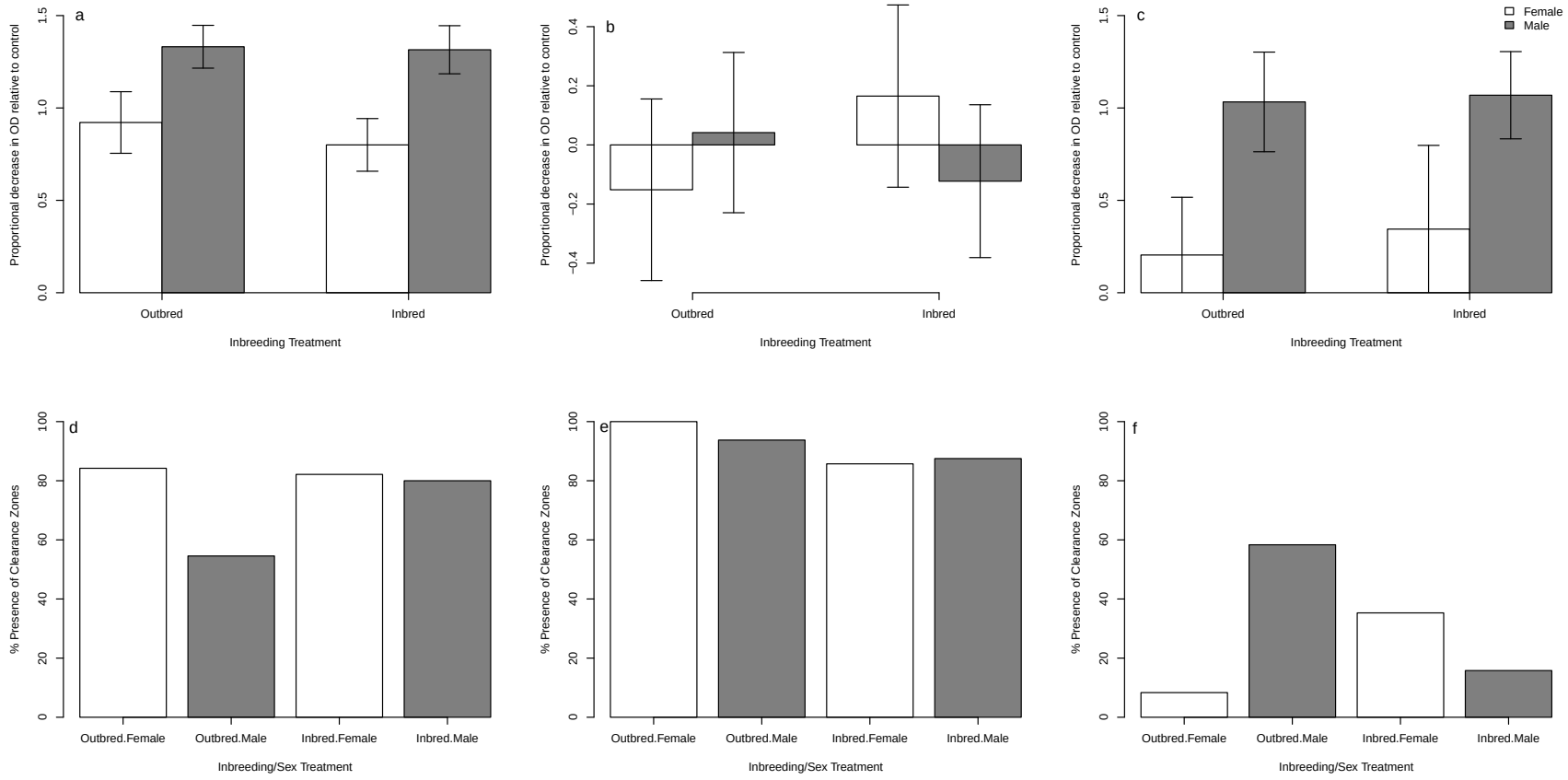

Figure 5.1: Effects of inbreeding on lytic activity (a-c) and bactericidal activity (d-f) of breeding male and female parents' *Nicrophorus vespilloides* secretions during egg laying (a & d), hatching (b & e) and dispersal (c & f). (a-c) Comparisons of proportional decline in optical density of a lysozyme substrate after 10 min relative to control (mean ± SE). (d-f) Comparisons of % presence of clearance zones after overnight incubation on *Bacillus subtillis*.

Table 5.2: Effects of inbreeding on the presence of bactericidal activity on *Bacillus subtillis* in breeding male and female parents' *Nicrophorus vespilloides* secretions during egg laying, hatching and dispersal. For each factor parameter estimates (Par) with standard error (SE), Z-values and *P* values compare outbred and inbred male and female parents and the effect of stage. n = 220.

Factor	Par	SE	Z-Value	*P*-Value
Inbreeding status (IS)	0.138	0.36	0.382	0.703
Sex	-0.254	0.36	-0.701	0.483
Stage (hatching)	1.423	0.55	2.6	0.009
Stage (dispersal)	-2.305	0.38	-6.08	<0.0001
Bacteria in secretions	2.159	1.05	2.05	0.04
IS * Sex	-0.62	0.72	-0.857	0.392
IS * Stage (hatching)	-2.332	1.31	-1.78	0.075
IS * Stage (dispersal)	-0.658	0.78	-0.846	0.397
Sex * Stage (hatching)	0.548	1.11	0.492	0.622
Sex * Stage (dispersal)	1.676	0.78	2.15	0.0318
IS * Sex* Stage (hatching)	13.329	1533.7	0.009	0.993
IS * Sex * Stage (dispersal)	-5.709	1.74	-3.27	0.001

Table 5.3: Effects of inbreeding on the presence of bactericidal activity on *Bacillus subtillis* on breeding male and female parents' *Nicrophorus vespilloides* secretions during egg laying, hatching and dispersal. For each factor parameter estimates with standard error, Z-values and *P*- values compare outbred and inbred female (n = 120) and male (n = 100) parents and stage.

	Female				Male			
Factor	Par	SE	Z-Value	*P*-Value	Par	SE	Z-Value	*P*-Value
Inbreeding status (IS)	0.362	0.52	0.69	0.488	-0.33	0.56	-0.592	0.554
Stage (hatching)	1.185	0.83	1.42	0.155	1.85	0.77	2.404	0.016
Stage (dispersal)	-2.956	0.56	-5.32	<0.0001	-1.587	0.55	-2.86	0.004
IS * Stage (hatching)	-16.626	2534.73	-0.01	0.99	-2.454	1.782	-1.38	0.17
IS * Stage (dispersal)	1.94	1.2	1.62	0.106	-4.135	1.36	-3.04	0.002

5.4 Discussion

I present evidence that the social immunity of male parents was affected by inbreeding, reducing the ability to regulate bactericidal activity at the dispersal stage of offspring development, whilst inbred and outbred female parents did not differ in their social immune functions. This evidence provides the first example of inbreeding effects on the social immunity of parents, mediated by lysozyme activity that improves offspring survival (Arce et al., 2012). I find strong differences between the lytic and bactericidal activity of the parents' secretions and no main effects between inbred and outbred parents. The effect of inbreeding on the presence of bactericidal activity depended on both the sex of the parent and the stage of offspring development. At the

Table 5.4: Effects of inbreeding on the strength of bactericidal activity on *Bacillus subtillis* on breeding male and female parents' *Nicrophorus vespilloides* secretions during egg laying, hatching and dispersal. For each factor, parameter estimates (Par) with standard error (SE), degrees of freedom, *t*-values and *P*- values compare outbred and inbred male and female parents and stage. n = 140.

Fixed Effect	Par	SE	Degrees of Freedom	t-Value	P-Value
Inbreeding status	0.011293	0.1493294	92	0.07562	0.9399
Sex	-0.069202	0.1504166	92	-0.46007	0.6466
Stage (hatching)	-0.202899	0.1476752	43	-1.37395	0.1766
Stage (dispersal)	-0.125327	0.2191242	43	-0.57194	0.5703

hatching and egg stage, inbred and outbred males did not differ but inbred males had lower presence of activity than outbred males during the dispersal stage, whilst inbred and outbred females did not differ throughout offspring's development. Below I discuss the implications of my results and further discuss the differences between the two assays, presence versus the strength of bactericidal activity and variation between sexes and stages.

There were no main effects of inbreeding on bactericidal and lytic activity, but inbreeding did affect the presence of bactericidal activity in males at the dispersal stage. There was no difference between inbred females and outbred females at any stage. The finding that females were unaffected by inbreeding is contrary to the prediction based on evidence that females have a higher immune function than males (Cotter and Kilner, 2010b; Steiger et al., 2011), that females would be more affected by inbreeding. Sex differences of inbreeding depression on immune responses have been shown previously and suggests that differences between males and females are due to differences in genetic architecture and dominance variation (Drayton and Jennions, 2011; Rantala and Roff, 2007). In *N. vespilloides* females have higher activity during the hatching stages, whilst males have higher protein levels at earlier and later stages of offspring development (Cotter and Kilner, 2010b). The compensation for the loss of a partner by males and the higher levels of immune proteins at early offspring development stages in males support the findings presented here that the social immune function of males varies compared to females (Cotter and Kilner, 2010b; Steiger et al., 2011).

The lytic activity during the hatching stage was highly variable around zero within all treatments and the mean OD was significantly lower than that at the egg and dispersal stages. The variation in OD around zero is likely to be caused by the fact that debris was not removed from lytic samples due to the small volumes of the individual samples. I observed such debris in the samples from the hatching stage in particular, a stage when parents are on the carcass are large proportion of the time,

and I suggest that changes in absorbance due to molecular breakdown may be obscured by the debris that would not be subject to such absorbance changes.

The presence of bactericidal activity was dependent on a three-way-interaction between inbreeding status, sex and stage. I found no effects of inbreeding status and stage in female parents, and only found that the presence of bactericidal activity was reduced in females at the dispersal stage, congruent with previous findings (Arce et al., 2012; Steiger et al., 2011). The separate analyses for males and females show that the effects of inbreeding in males were dependent on the stage of offspring development. There were no differences between inbred and outbred parents of either sex during the hatching stage. This is a surprising finding given that activity is highest at hatching and that males have lower lytic activity than females (Cotter and Kilner, 2010b; Steiger et al., 2011), leading to the prediction that inbreeding would have the strongest effect on females during the hatching stage. However, previous studies have not tested for the presence of bactericidal activity specifically (Cotter and Kilner, 2010b; Steiger et al., 2011). The lower presence of activity of the secretions of inbred males compared to outbred males is not as predicted because this is assumed to be a less significant stage for parents providing care to offspring (Arce et al., 2012). Male *N. vespilloides* commonly abandon the carcass and brood after hatching and therefore are often not present during the dispersal stage (Bartlett, 1988; Scott and Traniello, 1990). In addition, when larvae disperse from the carcass it is in a late stage of decomposition and protection of the carcass may be expected to be insignificant to larval growth. Whilst offspring at this stage gain little benefit in terms of feeding and growth from antimicrobial secretions that protect the carcass, they may still benefit from increased immunity to bacterial infection before dispersing from the carcass to pupate in the soil. However, this is not supported by a recent study on the secretions produced by the larvae themselves, in which bactericidal activity in larval secretions also decreases in function compared to previous stages (Arce et al., 2013). Previous studies find that social immune responses vary depending on sex and stage (Arce et al., 2012; Cotter and Kilner, 2010b; Steiger et al., 2011), representing a variable and complex regulation of social immunity, the inbreeding effects on social immunity reflect this complexity, as they depend on the sex of the parent and stage of offspring development.

The inbreeding effects on the bactericidal but not lytic immune functions suggest that the differences in inbreeding effects between the two functions lie in the costs associated with each (Arce et al., 2012). The variation in activity occurs in males who usually have lower activity and in the immune function with lower costs. The lytic and bactericidal activity of the secretions are a product of the same lysozyme protein (Arce et al., 2012). The lytic assay tests the primary function of the lysozyme to break down bacterial cell walls, whilst the bactericidal assay tests the function of the lysozyme to kill live whole bacterial cells (Arce et al., 2012; Masschalck and Michiels,

2003). The lysozyme therefore has both lytic enzymatic functions and bactericidal functions that kill cells by summed non-enzymatic and non-lytic functions such as muramidase hydrolysis and additional antimicrobial peptides (AMPs) (Arce et al., 2012; Masschalck and Michiels, 2003). The different activities of the one lysozyme protein may explain the temporal differences between lytic and bactericidal activity, and bacterial killing is suggested to have a lower metabolic cost at the latter stages of offspring development due to the build-up of lysozyme degradation products (Arce et al., 2012; Masschalck and Michiels, 2003). Evidence also suggests that the social immunity provided by parents is a complex suite of social immunity functions including additional components not measured in this study, and oral secretions that may provide further protection against other pathogens such as gram-positive bacteria (Arce et al., 2012; Degenkolb et al., 2011). I suggest that the differences in inbreeding effects between the two functions lie in the costs associated with each, in which the variation occurs for a lysozyme function with lower costs and in males that generally have lower activity than females. The reduction in future reproductive success in inbred parents may also lead to females and males investing more highly into the current brood, and so counteract physiological costs of inbreeding. As males rarely provide care during the dispersal stage, males may cease increasing investment at this stage of development. Measuring the effect of inbreeding on the personal immunity of parents whilst providing care, or the social immune functions during a successive brood, would test whether investment in these functions is reduced as a consequence of increasing investment due to inbreeding. This prediction is based on evidence that social and personal immune functions suffer a trade-off in functions in *N. vespilloides* (Cotter et al., 2013) and because parents increase social immune protection when the risk of death is increased (Cotter et al., 2010b).

I find variation in the effects of inbreeding on the bactericidal activity of secretions, showing effects on whether there is activity or not but no effects on the strength of activity when it is present. I note that whilst I found no effects of inbreeding status, sex or stage on the strength of bactericidal activity this may be due to a lack of statistical power (n=140 over three stages and four treatment groups). However this smaller sample size is as a consequence of a large number of samples that had no bactericidal activity, particularly at the dispersal stage and this absence is interesting in itself. The effects of inbreeding on the presence but not the strength of bactericidal activity suggests that the regulation of social immune function may have been affected. The activity of lysozyme is upregulated in *N. vespilloides*, as activity increases from a zero baseline when a carcass is attained (Arce et al., 2012; Cotter and Kilner, 2010b). The bactericidal activity of *N. vespilloides* secretions is suggested to derive from the properties of lysozyme and from AMP activation following lysozyme degradation (Arce et al., 2012; Masschalck and Michiels, 2003). The greatest variation in whether bactericidal activity is present occurs during the dispersal stage, when the

presence of bactericidal activity is particularly low. As previously argued, inbred males may avoid mounting a social immune response towards the end of offspring development at the cost of their own personal immunity (Cotter et al., 2013). However, evidence suggests that lytic activity presents higher physiological costs than mounting a bactericidal activity response (Arce et al., 2012) and inbred males do not reduce lytic activity during the dispersal stages. This therefore does not explain the reductions in bactericidal activity of inbred males during dispersal. I suggest that based on the finding that the presence of bactericidal activity was subject to stronger effects of inbreeding that the regulation of social immune functions were affected by inbreeding more than the strength of bactericidal activity.

The complex suite of mechanisms of the social immune response in *N. vespilloides* appear to be affected by inbreeding in males, but only in the later stages of offspring development. The lytic and bactericidal activity of secretions remains high during the most important stages of offspring development when both male and female parents are inbred. I suggest that possible causes for such effects of inbreeding may be due to the regulation of specific immune functions, and reductions in later stages of offspring development dependent on the costs of the social immune function relative to an individuals own personal immunity and their future reproductive opportunities.

6 Mate Choice As A Mechanism For Inbreeding Avoidance

Abstract

The significant fitness costs that inbreeding imposes may be expected to lead to the evolution of inbreeding avoidance by mate choice. A mate may be chosen both sequentially and simultaneously and the occurrence of each depends on how potential mates encounter one another. However, sequential and simultaneous mate choice differ in their costs and benefits, and so each strategy differs in the whether an individual will exert a preference for a particular mate. Appropriately testing for simultaneous or sequential mate choice is therefore necessary to account for the different forms of mating decisions and preferences that determine the costs of inbreeding avoidance. In this study I tested whether *Nicrophorus vespilloides* exhibit inbreeding avoidance through both sequential and simultaneous mate choice. Despite previous evidence that inbreeding depression reduces the fitness of potential offspring, I found no evidence that females avoided mating with a close relative when choice was both sequential and simultaneous. By considering the costs of pre-copulatory mate choice and the life-history of *N. vespilloides*, I conclude that investigations on mate choice need to further test the effects of relatedness on post-copulatory mate choice, taking into consideration the competition and opportunistic mating of males and females.

6.1 Introduction

Inbreeding is the mating between two related individuals resulting in an increase in homozygosity and often a reduction in fitness known as inbreeding depression (Crow and Kimura, 1970; Falconer and Mackay, 1996; Lynch and Walsh, 1998). Inbreeding depression affects a wide range of species (DeRose and Roff, 1999; Drayton et al., 2010; Miller et al., 1993) and the significant fitness costs that inbreeding imposes is expected to lead to the evolution of inbreeding avoidance (Blouin and Blouin, 1988). Inbreeding avoidance may occur through mate choice with a preference for an unrelated partner, sex-biased dispersal, extra-pair copulations (EPC) and other mechanisms (Blouin and Blouin, 1988; Pusey and Wolf, 1996). These mechanisms result in related individuals avoiding inbreeding by evolving a preference to only mate with unrelated partners, physically separating male and female relatives by dispersal (Nelson-Flower et al., 2012; Pusey, 1987) or having extra-pair copulations with a more unrelated individual than the social partner (Brouwer et al., 2011; Foerster et al., 2003; Pärt, 1996; Pusey and Wolf, 1996). Mate choice is a commonly studied mechanisms for inbreeding avoidance (Blouin and Blouin, 1988; Johnson et al., 2010; Lihoreau et al., 2007; Pusey and Wolf, 1996; Thomas and Simmons, 2011; Tien et al., 2011). Defined as the choice of a social or copulation partner, the choice of sperm for fertilisation, or the choice to invest differentially in particular zygotes; mate choice allows the choosing sex to benefit either directly from the provision of better resources or indirectly by increased genetic benefits (Kempenaers, 2007). A mate may be chosen sequentially or simultaneously and the occurrence of mate choice depends on how potential mates encounter one another (Barry and Kokko, 2010; Kempenaers, 2007).

Sequential mate choice is likely to commonly occur in situations where potential mates are encountered singly, in which females chose whether or not to mate with one potential mate (Barry and Kokko, 2010). In contrast, simultaneous mate choice is likely to occur in situations where multiple potential mates are encountered at the same time, and females chose a particular male over the other(s) (Barry and Kokko, 2010). Sequential and simultaneous mate choice differ in their costs and benefits, and so each strategy differs in the whether an individual will exert a preference for a particular mate. Appropriately testing for simultaneous or sequential mate choice is necessary to account for the different forms of mating decisions and preferences that determine the costs to inbreeding avoidance (Kokko and Ots, 2006). The evolution of inbreeding tolerance may be more likely when mate choice occurs sequentially as the number of future potential mates is dependent on the number of potential mates in a population (Bilde et al., 2005; Kokko and Ots, 2006). Not only should mechanisms for inbreeding avoidance be considered, such as mate choice or sex-biased dispersal, but within mechanisms such as mate choice, whether mate choice is sequential and

simultaneous must be considered to test the presence of inbreeding avoidance appropriately.

The evolution of particular inbreeding avoidance mechanisms is determined by the cost of the mechanism, the strength of inbreeding depression and the relatedness of potential mates (Kokko and Ots, 2006; Pusey and Wolf, 1996; Szulkin et al., 2013; Waser et al., 1986). For example, in the case of sequential mate choice which involves situations where individuals meet single potential partners, refusing a mate may be costly as there is no information on future mating opportunities and when mate encounter rates are low and when an individual's only mating opportunity is refused, impose a higher costs than that of inbreeding (Barry and Kokko, 2010). The evolution of inbreeding avoidance is influenced by the mate encounter rate, which in turn depends on the population structure, whether a sex is limited and the mating strategies of a species (Kokko and Ots, 2006). Hence, the cost of a particular inbreeding avoidance mechanism not only depends on the costs of inbreeding but on the life-history and population structure of a species (Kokko and Ots, 2006). The effects of life-history and population structure means that the evolution of particular inbreeding avoidance mechanisms are strongly linked with mating opportunities, mating strategies and the costs of mate choice, sex-biased dispersal and EPC. In the case of mate choice, the mating strategies are particularly important as this determines how individuals encounter one another and the criteria used for mating decisions (Kokko and Ots, 2006).

The burying beetle, *Nicrophorus vespilloides*, is an excellent study system to test the presence of inbreeding avoidance because inbreeding incurs substantial fitness costs (Mattey et al., 2013, Chapter 2). In addition, the life-history of the species provides the opportunity for potential mates to be related, because breeding adults search for and aggregate on small vertebrate carcasses to reproduce (Eggert and Müller, 1997; Scott, 1998). To test for inbreeding avoidance by pre-copulatory mate choice, I considered mate choice in the context of the two mating strategies of *N. vespilloides* (Eggert, 1992). First, males without carcass emit a pheromone to attract females for a single mating, after which the female stores sperm from the male for future reproductive opportunities (Bartlett, 1987b; Eggert, 1992). This strategy involves the female sequentially choosing between potential mates, making a decision as to whether or not to mate with a single male of a given relatedness, in the absence of information on the relatedness of future potential mates (Barry and Kokko, 2010). Second, both males and females search for a carcass on which to reproduce, and once a carcass is found by both a male and a female they mate multiple times to produce a brood on the carcass (Eggert and Müller, 1997; Müller and Eggert, 1989; Scott, 1998). If more than two males (or females) find the carcass, this results in competition and the potential for simultaneous choice between two males by the female (Eggert, 1992). For

the second strategy mate choice experiments in *N. vespilloides* should account for the fact that females often mate multiple times and with multiple males, though provide care with only one male. The frequency with which the female mates with each male will provide more detailed information regarding the female's preference, rather than a single mating event or which male the female first mates with, which is unrepresentative of the proportion of the brood the male sires (Pettinger et al., 2011).

In this study I tested whether *N. vespilloides* exhibit inbreeding avoidance through both sequential and simultaneous mate choice. First, I recorded whether males and females copulated in trials in which the female was either related or unrelated to the male. This design tested for inbreeding avoidance via sequential mate choice and simulated mate choice decisions in the absence of a carcass. Given that males are likely to encounter females up to two times a day in the absence of a carcass (Eggert and Müller, 1989), avoiding a related mate is likely to impose a low cost in relation future breeding attempts, as females are likely to encounter other males of a different relatedness. Hence, I predicted that more females would mate with unrelated males than females paired with related males. Second, I provided females with a related male and an unrelated male to test for simultaneous mate choice in the presence of a carcass. Given that females regularly mate with multiple males on a carcass and can control the proportion of offspring each males sires by controlling the number of copulations (Eggert, 1992; Pettinger et al., 2011), the costs of inbreeding depression are high (Mattey et al., 2013), and I prevented males from interacting and competing with one another, I predicted that females would mate more frequently with the unrelated male.

6.2 Materials and Methods

6.2.1 General Methodology

All beetles used in the experiments were from a large outbred laboratory population maintained at the University of Edinburgh. General husbandry procedures and the maintenance of outbreeding in the laboratory stock population were as outlined in Chapter 2. The population used in the experiment comprised of 5th to 10th generation beetles originally collected at Corstorphine Hill, Edinburgh; 2nd to 4th generation beetles collected from Hermitage of the Braid, Edinburgh; 3rd generation beetles collected at Jodrell Bank, Manchester; 6th and 7th generation beetles collected at Kennel Vale, Cornwall and 1st generation beetles collected at Madingley Woods,

Cambridge. Only virgin beetles were used in the experiments. Copulation was confirmed when it was observed that the male's adeagus had been inserted into the female's vagina (House et al., 2008). For all trials, the size of both males and females was recorded by measuring the width of the individual's pronotum using digital callipers, in order to account for a potential mating preference based on either male size or female size.

6.2.2 Sequential Mate Choice

To test for sequential mate choice, I tested the mating behaviour of a female paired with either a related or unrelated male. Fifty experimental pairs were setup, all pairs were placed in a petri dish (90mm diameter, and 12mm high) and the time it took for the first copulation was recorded (Figure. 6.1a). If the pair did not mate within the 30 minute trial period the mating was recorded as unsuccessful, based on pilot data I collected, this reliably predicts whether a pair will mate within an hour. Related males and females were full siblings whilst unrelated individuals did not share a common grandparent or a closer relative.

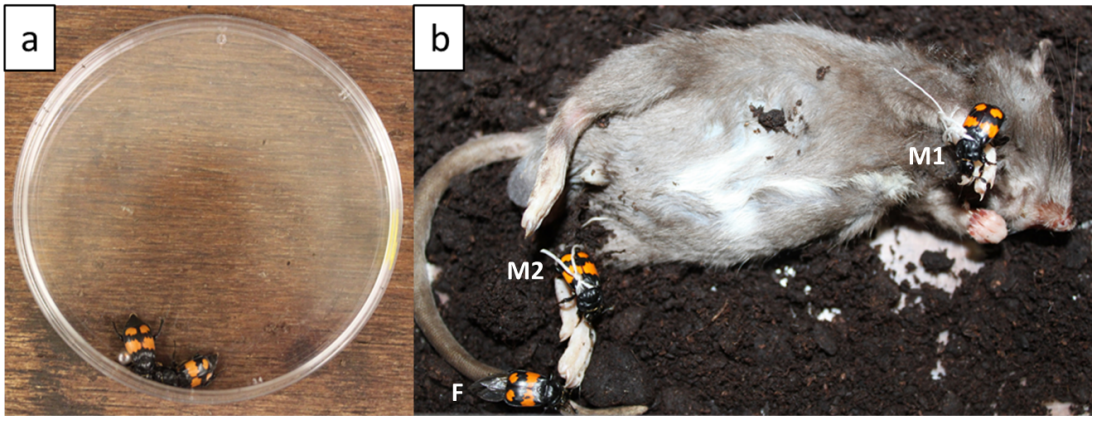

Figure 6.1: Experimental design to test for sequential (a) and simultaneous (b) mate choice in *Nicrophorus vespilloides*. (a) All pairs were placed in a petri dish and whether copulation was successful within 30 min and the time it took for the first copulation was recorded. (b) Males were separated from one another by attaching each male to opposite ends of the carcass. The first male (M1) was attached to the right foreleg of the carcass. The second male (M2) was attached to the left hind leg of the carcass, to prevent the two males competing or interacting with one another. The female (F) was placed at centre of the carcass and the time the female first contacted each male and the time of each copulation within 60 min was recorded.

6.2.3 Simultaneous Mate Choice

To test for simultaneous mate choice I recorded the number of times that a female copulated with either a related or unrelated male. Trials consisted of a male that was unrelated to the female (not sharing a common grandparent or closer relative) and a male that was related to the female (a full sibling, F=0.25). Trials were setup in transparent plastic boxes (17cm x 12cm and 6cm high) by providing 0.5cm of moist soil and a previously frozen rat carcass (supplied from Livefoods Direct Ltd, Sheffield, UK). Thirty choice trials were setup and three trials were excluded because a male escaped. The carcass had a mass range from 41 to 82g, which had no effect on the number of copulations (Z = -0.32, $P = 0.75$). This larger carcass size allowed for a distance between the two males where they could not interact with one another, preventing intra-sexual competition that could potentially influence access to the female or female mating decisions. Males were separated from one another by attaching each male to opposite ends of the carcass. The first male was attached to the right foreleg of the carcass by dental floss tied around the male's pronotum. The second male was attached as the first but to the left hind leg of the carcass. It was ensured that each male could move by providing each 2cm of give so that normal mating behaviour was not obstructed (Figure. 6.1b). Related and unrelated males were alternated between the foreleg and hind leg of the carcass. The female was placed at the centre of the carcass and I recorded the time the female first contacted each male and the time of each copulation within 60 min.

6.2.4 Statistical Analyses

All statistics were carried out in R version 2.13. In the analysis of the sequential mate choice trial, I first tested whether a mating was successful or not and whether the occurrence of successful trials was affected by the relatedness of the male. All final models were based on the lowest AIC score, if a difference in the AIC score was less than two then the simplest model was chosen. To test for an effect of relatedness on whether the male mated successfully, I used a generalised linear model fitted with binomial errors, fitting the relatedness of the pair (unrelated or related), male size and female size. Male size and female size had no significant effect on whether the pair mated or not (Z = -1.53, $P = 0.13$ and Z = -1.48, $P = 0.14$, respectively), and these terms were not included in the final model based on AIC scores. Second, I tested for an effect on the time it took copulate between related and unrelated males for all successful trials in which the pair mated. To test for an effect of pair relatedness on time to copulation I used a generalised linear model fitted with quasi-Poisson errors to account for over-dispersion of the data, and fitted the relatedness of the male

(unrelated or related), male size and female size as fixed factors. Time to copulate was not affected by either male size or female size (Z = -1.68, $P = 0.1$ and Z = -1.89, $P = 0.067$, respectively), and these variables were not included in the final model based on AIC scores.

In the analysis of the simultaneous mate choice trial I tested whether the frequency of copulations for each male was affected by whether the female was related or unrelated to the male. To do this I used a generalised linear mixed model fitted with Poisson errors, fitting the relatedness of the female (unrelated or related), male size, whether the male came into contact with the female first (yes or no) and the position of the male on the carcass (foreleg or hind leg). In all models female identity was added as a random effect to account for the non-independence between males in the same trial. An interaction between male size and relatedness had no significant effect on the frequency a male copulated (Z = 0.44, $P = 0.73$ and Z = -1.4, $P = 0.16$, respectively), and these terms were not included in the final model based on AIC scores.

6.3 Results

6.3.1 Sequential Mate Choice

Within 30 min 41 of 60 pairs copulated and there was no significant difference between related and unrelated males: 76% of unrelated males mated with the female, whilst 88% of the related males mated with the female ($Z_{48} = 1.091$, $P = 0.28$) (Figure 6.2a). In addition, related and unrelated pairs that did copulate did not differ significantly in the amount of time it took to copulate ($Z_{39} = -0.021$, $P = 0.98$) (Figure 6.2b).

6.3.2 Simultaneous Mate Choice

Relatedness between a male and female had no effect on the frequency with which they copulated (Z = 0.994, $P = 0.321$) (Figure. 6.3a). However, males attached to the foreleg of the carcass copulated significantly more than males attached to the hind leg of the carcass (Z = -2.41, $P = 0.016$) (Figure. 6.3b). There was a non-significant trend that males who encountered the female first, copulated more frequently than males who encountered the female second (Z = 1.7, $P = 0.0898$). Therefore, I found no evidence of simultaneous mate choice based on the relatedness of pairs.

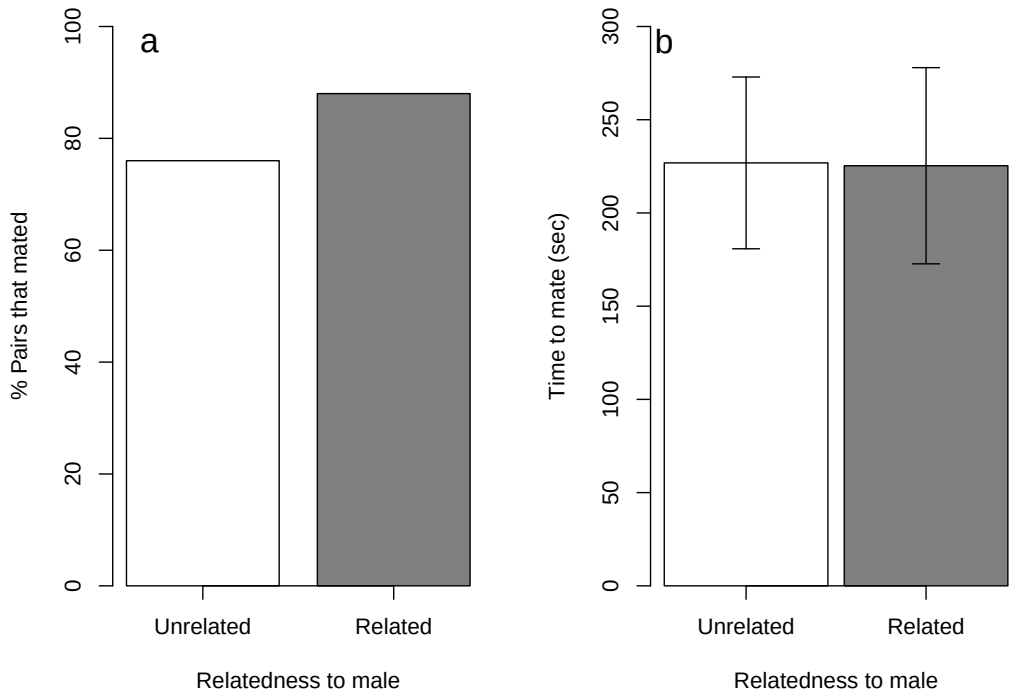

Figure 6.2: Effects of male relatedness on sequential mate choice inbreeding avoidance in *Nicrophorus vespilloides*. Comparisons of unrelated males (white bars) or related males (grey bars) paired with a female on the percentage of pairs that mated (a), and for those that did mate the time to copulation (b) (mean ± SE).

6.4 Discussion

I found no evidence that females avoided mating with a close relative in *N. vespilloides*. Females showed no mating preference for unrelated males over related males when choice was sequential or simultaneous in the presence of a carcass. Testing for both sequential and simultaneous mate choice accounted for the two pre-copulatory mating strategies of *N. vespilloides*, however I present evidence that females do not make mating decisions based on the relatedness of the potential mate. Below I discuss the implications of these results and whether inbreeding avoidance has evolved through other mechanisms, or whether it is not beneficial to evolve inbreeding avoidance through pre-copulatory mate choice in *N. vespilloides*.

I found that females did not choose mates based on relatedness in the context of either of the two mating strategies in *N. vespilloides*. Female *N. vespilloides* regularly encounter individual males up to twice a day in the absence of a carcass (Eggert, 1992; Eggert and Müller, 1989), and females make a decision to mate with a male or not.

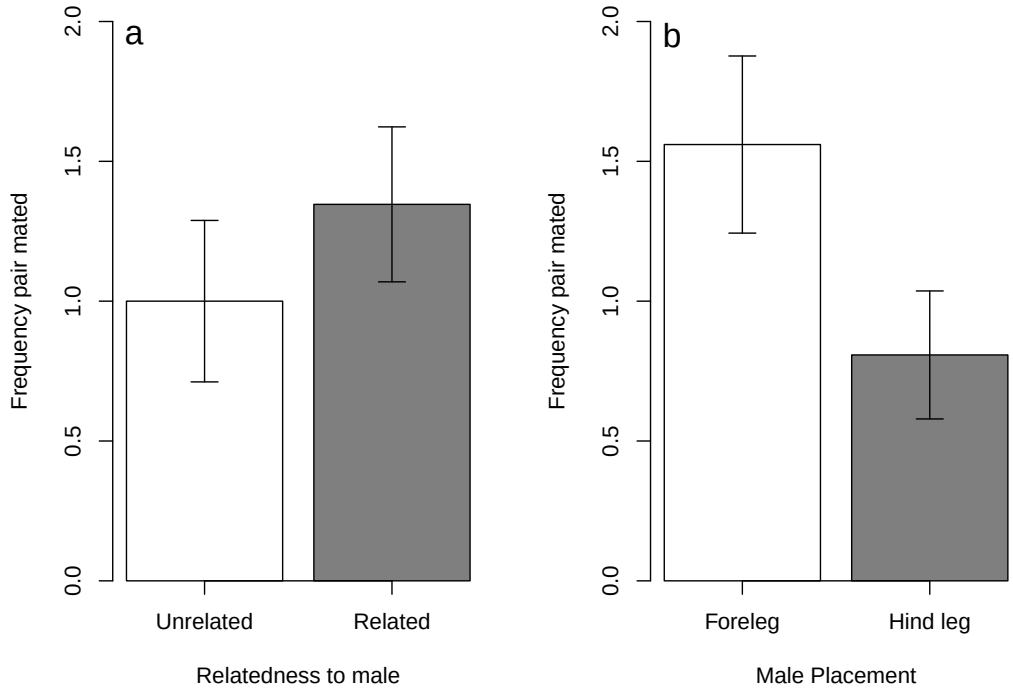

Figure 6.3: Effects of male relatedness (a) and male placement (b) on simultaneous mate choice in *Nicrophorus vespilloides*. (a) Comparison of unrelated males (white bars) or related males (grey bars) on the number of copulations with the female (mean ± SE). (b) Comparison of males placed at the foreleg of the carcass (white bars) and the hind leg of the carcass (grey bars) on the number of copulations with the female (mean ± SE).

However, I show here that females do not take the relatedness of the male into account. Second, females mate multiple times with multiple males in the presence of a carcass which often attracts numerous males and females to the reproductive opportunity (Eggert and Müller, 1997; Müller and Eggert, 1989; Scott, 1998). On a carcass, females often mate with more than one male (Bartlett, 1988), and so are unlikely to make a single mating decision to mate with only one particular male. Instead, females make a decision on which male to mate with more frequency and which male to sire a larger proportion of offspring (Pettinger et al., 2011). Here, I show that relatedness of either males were not used as criteria in simultaneous female mate choice. Understanding female mating strategies and decisions is vital to correctly test the presence of pre-copulatory mate choice. I predicted that females would avoid inbreeding using pre-copulatory mate choice due to the high costs of inbreeding in *N. vespilloides* (Mattey et al., 2013, Chapter 2). Given that there is no mating preference for unrelated males, the high costs of inbreeding depression and the high relatedness of potential mates provided to females, I therefore suggest that the evolution of

pre-copulatory mate choice might represent too high a fitness cost to females relative to the severity of inbreeding depression and the likelihood of inbreeding.

Whether the life-history of *N. vespilloides* leads to high costs of pre-copulatory mate choice depends on mate encounter rates, the costs of kin recognition and the likelihood of inbreeding. Both males and females compete with members of their own sex to breed on a carcass (Bartlett, 1988), resulting in the successful, dominant pair breeding on the resource (Eggert and Müller, 1997; Trumbo, 1992). In this situation, I suggest that neither males or females are able to exert any choice for their social partner due to the strong influence of competition on breeding success. In addition to competition, satellite males also mate on the carcass (with the breeding female or another female) and other females parasitise the brood by laying eggs (Eggert, 1992). Hence, mating events may not rely on mate choice decisions but may be instead determined by competition and opportunistic mating strategies. Results from the simultaneous mate choice trial support the opportunistic nature of mating because males attached to the foreleg of the carcass copulated with the female more than the male attached to the hind leg. This higher number of copulations is unlikely to represent a preference for males positioned at the foreleg but instead is likely to be due to the carcass preparation behaviour of the female. During the observations in this study, I observed that females spent more time preparing the carcass at the head than at the tail, and therefore spending more time closer to the male attached to the foreleg. Therefore, males attached to the foreleg were in contact with the female more than the male attached to the hind leg, with more opportunities to mate with the female. This observation of carcass preparation behaviour suggests that the preference for males attached to the foreleg was as a result of mating opportunistically, in the absence of male-male competition.

Inbreeding avoidance by mate choice may not be present in *N. vespilloides* due to the significant costs of kin recognition, a mechanism that requires genetic information and self-reference (Mateo and Johnston, 2000; Weddle et al., 2013). Cuticular hydrocarbons (CHCs), the major histocompatibility complex (MHC), and other mechanisms such as hormones may provide information on the relatedness of unfamiliar individuals (Gasparini and Pilastro, 2011; Howard and Blomquist, 2005; Lihoreau et al., 2007; Thomas and Simmons, 2011; Tregenza and Wedell, 2000). Chemical information on relatedness is provided by CHCs in several insect species, particularly social insects (Howard and Blomquist, 2005; Tsutsui, 2004; Weddle et al., 2013). In *N. vespilloides*, CHCs provide information on breeding status and sex, though there is no evidence of individual recognition (Müller et al., 2003; Steiger et al., 2009). Evidence from Chapter 3 on how individuals respond to inbred partners suggests that partners were able to distinguish between inbred and outbred individuals, suggesting that genetic information may be conferred between potential

partners, though there is no evidence of an ability to self-reference. The absence of pre-copulatory mate choice based on relatedness suggests that the costs of this mechanisms may be too high relative to the costs and likelihood of inbreeding. The absence of pre-copulatory mate choice might be because matings are opportunistic, because the cost of kin recognition is too high, because the probability of inbreeding is too low or because inbreeding is avoided via another mechanism.

Inbreeding avoidance may occur by another mechanism that is less costly than pre-copulatory mate choice. In the absence of pre-copulatory mate choice the opportunistic mating of *N. vespilloides* suggests that another likely mechanism for inbreeding avoidance might be post-copulatory mate choice. Reproducing females require a suitable carcass that successfully attracts males and that they can successfully raise a brood on, termed a 'bonanza' resource which is unpredictable (Eggert, 1992; Scott, 1998). The mating opportunities in the absence of a carcass and the storage of sperm until a suitable carcass is attained (Bartlett, 1987b; Eggert, 1992) provides females with the potential for post-copulatory mate choice, i.e. choosing sperm provided by an unrelated male over a related male. Females also mate multiply once a carcass is attained and so have additional opportunities to fertilise sperm from a more unrelated male, thereby allowing the unrelated male to sire a larger proportion of the offspring (Pettinger et al., 2011). Post-copulatory choice of unrelated sperm is found in the field cricket *Teleogryllus* (Bretman et al., 2009; Tregenza and Wedell, 2000). In addition, evidence of post-copulatory mate choice is found in *N. vespillodies*, based on male dominance (Pettinger et al., 2011). In this study dominant males sired proportionally fewer offspring relative to subordinate males than would be expected of the number of times dominant and subordinate males mated with females (Pettinger et al., 2011). The study also found that dominant males sired proportionally fewer offspring in the absence of a carcass than in the presence (Pettinger et al., 2011). I therefore suggest that the presence of post-copulatory mate choice of unrelated mates is investigated to test for inbreeding avoidance in *N. vespilloides*.

Extra-pair copulation is also a possible mechanism for inbreeding avoidance, though individuals may gain little from a small number of extra-pair matings without post-copulatory mate choice, as single matings in the presence of a carcass may not transfer enough sperm to fertilise eggs that hatch successfully or sire a large proportion of the offspring (Eggert and Müller, 1997; Pettinger et al., 2011; Trumbo, 1992). However, EPCs with brood parasitising females and satellite males should be investigated as a possible mechanism for inbreeding avoidance in conjunction with post-copulatory mate choice, because this allows the choice of more unrelated sperm from opportunistic extra-pair matings on the carcass. Sex-biased dispersal is a costly mechanism for inbreeding avoidance (Waser et al., 1986). In *N. vespillodies* (where the dispersal behaviour is largely unknown) both males and females search for a carcass,

and both sexes aggregating to compete for the resource (Eggert and Müller, 1997; Scott, 1998). This means that both sexes with a high relatedness may still be likely to encounter one another even after dispersal from the resource. Inbreeding avoidance in this species cannot be ruled out by pre-copulatory mate choice alone, and the presence of post-copulatory mate choice and EPC should be further investigated.

Though the mechanism of inbreeding avoidance by pre-copulatory mate choice may not be present in *N. vespilloides*, it may be beneficial to have no preference based on mate relatedness. The evolution of inbreeding avoidance depends on the costs and benefits of inbreeding and how these counteract the costs and benefits of the mechanisms to avoid inbreeding (Waser et al., 1986). Benefits to inbreeding include the increase of a parent's inclusive fitness, avoiding outbreeding depression, the purging of deleterious alleles and reducing sexual conflict when providing biparental care (Kokko and Ots, 2006; Pusey and Wolf, 1996; Rankin, 2010; Szulkin et al., 2013; Thünken et al., 2007). For inbreeding avoidance to evolve, the benefit to discriminating against a relative and avoiding mating opportunities are predicted to provide a higher fitness benefit than not discriminating between kin; this benefit is determined by the magnitude of inbreeding depression and by the relatedness of the potential mate (Waser et al., 1986). Mate choice may be a costly mechanism for reproductive opportunities that are unpredictable, as in *N. vespilloides* (Kokko and Ots, 2006). While inbreeding depression is substantial in *N. vespilloides* (Mattey et al., 2013), these costs do not account for any potential benefits that have not been investigated. As well as increasing inclusive fitness, inbreeding may also incur social benefits. For instance, the African cichlid fish *Pelvicachromis taeniatus*, preferentially mates with kin, this preference is associated with increased levels of cooperation when providing bi-parental care (Thünken et al., 2007). *Nicrophorus vespilloides* does provide biparental care (Eggert et al., 1998; Smiseth and Moore, 2004a), and whilst there is no evidence of inbreeding avoidance, I present no evidence of preference for related kin. The inbreeding history of the population of *P. taeniatus* describes a bottleneck whereby deleterious effects of inbreeding were likely purged, leading to low inbreeding depression, unlike what is found in *N. vespilloides* (Mattey et al., 2013; Thünken et al., 2007, Chapter 2). It is noted that the difference in life-histories and population structures provides context for the evolution of active inbreeding in two species that provide biparental care. Whilst I found no evidence for preferential inbreeding in *N. vespilloides*, any benefits to tolerating inbreeding are untested in this system, and so the net costs of inbreeding are potentially unknown. Whether inbreeding is tolerated cannot be determined until other mechanisms for inbreeding avoidance are investigated, there is more information about the occurrence of inbreeding in the wild, and any potential benefits such as increased cooperation between relatives are investigated.

To conclude, I find no evidence of pre-copulatory mate choice for unrelated males, despite previous evidence that inbreeding depression reduces the fitness of potential offspring (Mattey et al., 2013, Chapter 2). It is possible that inbreeding avoidance has not been detected by the appropriate mechanism: I suggest post-copulatory mate choice as the most likely mechanism to avoid inbreeding because it accounts for both mating strategies of females and the opportunistic nature of mating decisions. It is also possible that inbreeding may be tolerated, either because in the wild it is at substantially low levels, or because some benefits are gained from inbreeding. By considering the costs of pre-copulatory mate choice and the life-history of *N. vespilloides*, I conclude that investigations on mate choice need to investigate the effects of relatedness on post-copulatory mate choice, taking into consideration the competition and opportunistic mating of males and females.

7 General Discussion

I present evidence that *Nicrophorus vespilloides* is subject to strong direct, intergenerational and social effects of inbreeding on parent and offspring traits. Direct effects of inbreeding describes traits of inbred individuals that are affected by the individual's own inbreeding status. Direct effects of inbreeding may affect non-social traits such as growth and survival, and also social traits such as social behaviours and social immunity. In the case of social traits, inbreeding may also lead to indirect effects of inbreeding, in which an individual is affected by the inbreeding status of another individual with whom they interact with or depend on. Intergenerational effects are a class of indirect inbreeding effects where inbred parents typically reduce the fitness of outbred offspring. Here, I define social effects of inbreeding to include direct and indirect effects of inbreeding on social traits and indirect effects of inbreeding. First, I found evidence for strong inbreeding depression, as inbred offspring had reduced survival at several stages of development (Chapter 2). Furthermore, I show direct effects of inbreeding on social traits. Inbred offspring begged less than outbred offspring (Chapter 4), inbred male parents provided more care than outbred male parents (Chapter 3), inbred female parents provided less indirect care than outbred female parents (Chapter 4) and the secretions that inbred male parents produced had lower bactericidal activity than outbred male parents (Chapter 5). The reductions in such social traits not only suggest fitness costs to those individuals but that such traits may indirectly affect the fitness of other individuals within the family. Second, I present evidence of strong intergenerational effects of inbreeding. I show that the survival of outbred offspring raised by inbred female parents was reduced during the parental care stage of offspring development (Chapter 2). While my results and those of other studies suggest that such effects may be due to inbred parents providing poorer care to offspring (Mattey et al., 2013; Reid et al., 2003; Szulkin et al., 2007, Chapter 2), I show that the consequences of inbreeding in parents appear to be far more complex. Indeed, I present evidence that the inbreeding status of both individuals involved in a social interaction affected the outcome of the interaction, evidenced by a statistical interaction between the two (Chapter 3 and 4). Social effects such as these are predicted to affect the fitness of both individuals. My results on the consequences of inbreeding within a family further emphasises the need to consider the potentially complex social effects of inbreeding.

7.1 Social Effects of Inbreeding

I found that inbreeding had strong social effects on a species that provides biparental care, affecting begging by inbred offspring (Chapter 4), care by inbred parents (Chapter 3 and 4), increasing the care provided by parents when a partner or offspring were inbred (Chapter 3 and 4) and reducing the social immunity of inbred male parents (Chapter 5). Here, I focussed on the social effects within a family, a 'miniature' animal society that represents a social group of individuals whose fitnesses are largely dependent on one another (Cotter and Kilner, 2010a; Royle et al., 2002b). Within such a group, I provide evidence for social effects of inbreeding on individuals across generations, social interactions between individuals, and the social immunity provided to offspring by parents.

7.1.1 Are Intergenerational Effects of Inbreeding Associated with Parental Care?

I found strong intergenerational effects of inbreeding in which outbred offspring of inbred female parents had lower survival during the parental care stage of development (Chapter 2). Intergenerational effects of inbreeding are important because the mechanisms are often hypothesised to be associated with parental care (Reid et al., 2003; Szulkin et al., 2007). However, my results suggest that the reductions in offspring survival were not explained by the amount of care inbred parents provided, or by the properties of secretions that inbred parents applied to the carcass (Chapter 5). In particular, I predicted that the strong reduction in the survival of outbred offspring from hatching to dispersal when cared for by inbred female parents (when male parents were outbred) would reflect a reduction in direct care provided by inbred female parents when mated with an outbred male (Mattey et al., 2013, Chapter 2). However, offspring raised by one inbred parent actually received more direct care than when raised by two outbred parents (Chapter 3). My results suggest that the intergenerational effects of inbreeding are not explained by the amount of care provided by parents. Despite a number of studies that find intergenerational effects of inbreeding on the fitness of outbred offspring or grandoffspring (Mattey et al., 2013; Reid et al., 2007; Richardson et al., 2004; Szulkin et al., 2007), no studies to date provide evidence that such reductions in fitness are due to the care provided by parents (Mattey et al., 2013; Richardson et al., 2004, Chapter 2). This is supported by the intergenerational effects of inbreeding in the Seychelles warbler, *Acrophalus sechellensis*. In this study, outbred offspring were cross-fostered, and whilst increasing maternal homozygosity was associated with a reduction in offspring fitness, foster parent homozygosity was not (Richardson et al.,

2004). The authors suggest that the cross-fostering of *A. sechellensis* offspring and the reduction in offspring fitness associated with maternal homozygosity may be caused by maternal effects, where inbred female parents reduce their investment in eggs (Richardson et al., 2004). This is also suggested as a possible explanation in the song sparrow, *M. melodia* (Reid et al., 2003). The influence of inbreeding on maternal effects may explain the maternal-specific intergenerational effects of inbreeding in *N. vespilloides* (Mattey et al., 2013, Chapter 2), in which outbred offspring survived less well when raised by inbred female parents, but there was no difference in outbred offspring raised by inbred and outbred males (Mattey et al., 2013, Chapter 2). Maternal effects may also account for the strong reductions in outbred offspring survival in natural broods (Mattey et al., 2013, Chapter 2) but that mixed broods comprising of offspring produced by both inbred and outbred parents did not differ in survival, (this difference may also be due to environmental effects, discussed below). The mechanisms for intergenerational effects of inbreeding therefore still warrants further investigation, particularly the maternal effects from inbred female parents to outbred offspring such as investment in eggs. My results provide no evidence that the intergenerational effects of inbreeding in *N. vespilloides* are explained by the care provided by parents, and I suggest that other mechanisms may be responsible for the reduction in survival of outbred offspring raised by inbred female parents.

7.1.2 Effects of Inbreeding on Social Interactions

I show that social interactions between two individuals within a family were strongly affected by inbreeding: between two caring parents (Chapter 3) and between offspring and parents (Chapter 4). First, the inbreeding status of the parent had a significant effect on the amount of care provided by the partner, regardless of the amount of care provided by the parent (Chapter 3). This evidence suggests that each used a 'sealed bid' to determine how much care to provide (Chapter 3). The amount of care female parents provided also depended on the inbreeding status of both the male and the female, as evidenced by a statistical interaction (Chapter 3). This interaction describes that the compensation of care by a female parent when mated to an inbred partner was dependent on whether the female herself was inbred or outbred (Chapter 3). Second, inbreeding influenced communication between parents and offspring. Parents appeared to determine the amount of care to provide inbred offspring from cues other than begging, that are associated with the offspring's inbreeding status (Chapter 4). This is shown by the finding that parents provided more direct care to inbred offspring, despite inbred offspring begging less than outbred offspring (Chapter 4). As found in Chapter 3, the amount of care female parents provided to offspring depended on the inbreeding status of both individuals involved in the interaction, as

shown by a statistical interaction. This interaction describes that female parents only provided more direct care to inbred offspring when the female parent was outbred (Chapter 4). The statistical interactions present for female direct care (Chapters 3 and 4) affected two forms of social interactions by inbred females, and suggests that cooperation and communication of female parents were themselves affected by inbreeding (Chapter 3 and 4). However, the statistical interaction between the inbreeding status of two individuals was not consistent for both forms of care. In the provision of indirect care, an interaction between the inbreeding status of the male and female parent was also present, but inbred female parents instead provided more indirect care when mated to an inbred male partner, whilst outbred females did not differ in the amount of indirect care provided when mated to an inbred and outbred male partner (Chapter 3). This latter interaction on indirect care therefore suggests that the effects of inbreeding on social interactions and their consequences for parental care are complex and dependent on the type of care that parents provide. In conclusion, inbreeding appears to affect the assessment of a partner and reception of information from offspring in female parents. Few studies have investigated such interactions (Eklund, 1996) and none involving cooperative interactions. In addition to individuals in an interaction responding to an inbred individual leading to indirect effects of inbreeding, inbreeding in both individuals may in turn affect the response.

7.1.3 Social Effects of Inbreeding and The Environment

I show that the anal secretions of inbred male parents were less effective at killing *Bacillus subtillis* bacteria than the secretions of outbred parents, but that such effects were only significant during the dispersal stage of offspring development (Chapter 5). It is unclear whether these effects at a late stage of offspring development impact the offspring's environment significantly, and in turn the offspring's development. The secretions that parents apply to the carcass protect the resource from microbial competitors and the offspring from pathogen infection (Rozen et al., 2008). Whilst protection of the resource is likely to have little impact on offspring growth when offspring are dispersing away from the carcass, the effects of inbreeding on the social immunity of parents may still have an effect on the number of pathogens present in the environment, as larvae disperse into the soil to pupate, via the secretions they apply. Social immunity associated with parental care is present in a number of systems (Cardoza et al., 2006; Cotter and Kilner, 2010a; Fleming et al., 2009; Giacomello et al., 2006), and whilst other studies have investigated the effects of inbreeding on personal immunity (Calleri et al., 2006; Drayton and Jennions, 2011; Rantala and Roff, 2007; Reid et al., 2007; Townsend et al., 2010; Vitikainen and Sündstrom, 2011), whether social immune functions are reduced in inbred parents

have not been previously investigated. In *N. vespilloides* there is little evidence to suggest whether there are fitness consequences of social immunity at the dispersal stage of offspring development (Arce et al., 2012; Rozen et al., 2008). I therefore suggest investigating the effect of the parent's secretions on the eclosion success of offspring at the dispersal to clarify the effects of inbreeding on the social immunity at the dispersal stage. In conclusion, there is little evidence that inbred parents reduce the quality of the carcass which protects the offspring's environment and resource, however it remains unclear whether there is an impact on offspring from a reduction in inbred male parent's social immunity during dispersal.

I found that offspring survival in small artificial broods raised by inbred female parents did not differ from the survival of broods raised by outbred female parents (Chapter 3). In contrast, larger natural broods raised by inbred parents survived less well than those raised by outbred female parents (Chapter 2). The difference in survival between experiments suggests that weaker sibling competition may be present in smaller broods, providing a less harsh environment that buffered the intergenerational effects of inbreeding. Furthermore, inbred female parents provided less indirect care than outbred female parents when the male partner was absent, whilst inbred female parents provided as much indirect care as outbred female parents provided when the male partner was present (Chapter 3 and 4). Hence, the indirect care provided by inbred female parents with a partner also appear to be buffered to inbreeding effects. Therefore, I suggest that the severity of inbreeding effects were further increased by a harsher social environment within a family. Environmental effects may compound the effects of inbreeding which tends to occur in populations which experience harsher environments (Armbruster and Reed, 2005; Crnokrak and Roff, 1999; Fox and Reed, 2011; Hedrick and Kalinowski, 2000; Kristensen et al., 2006). For example, food limitations and the presence of competitors further increases inbreeding depression in traits such as survival (Enders and Nunney, 2010). Evidence in meerkats, *Suricata suricatta*, provides support for the social environment buffering the severity of inbreeding depression (Nielsen et al., 2012). The authors show that inbred and outbred pup survival do not differ, but when helpers discontinue provisioning at the juvenile stage inbred juveniles survive less well than outbred juveniles (Nielsen et al., 2012). The authors propose that the environment for offspring without helpers represents a harsher social environment that exacerbated the effects of inbreeding (Nielsen et al., 2012). In addition, interactions between inbreeding and the social environment of the European earwig, *Forficula auricularia*, show that inbreeding depression depends on the presence or absence of female parents (Meunier and Kölliker, 2013). My evidence that the absence of the male parent in *N. vespilloides* exacerbated inbreeding depression would be in contrast to previous studies that shows no costs for female parents when the male parent is absent (Smiseth et al., 2005). In light of the effects of inbreeding, I suggest that in an outbred family, there is no cost to females when male

parents are absent. However, inbred female parents providing care in the absence of male parents reduce the amount of indirect care provided as a consequence of physiological constraints of inbreeding, whilst still providing similar levels of direct care. In *N. vespilloides* both parents and offspring rely on the social environment of other members of the family, as their presence or absence affects growth, survival and the amount of care provided. Therefore, I suggest that future work should investigate how inbreeding interacts with the social environment in a family context.

Sibling competition might not only increase the effects of inbreeding, but may also be affected by inbreeding itself, and I suggest future work investigates the effects of inbreeding on competition between siblings. There is evolutionary conflict between siblings over the amount of resources parents should invest in each, and it is under strong selection (Mock and Parker, 1997; Parker et al., 2002). As with other social interactions within the family, social traits may be directly and indirectly affected by inbreeding, determining the amount of resources each sibling receives. The effects of inbreeding would be expected to affect the provisioning offspring receive as it has been shown that the presence of parents exacerbates sibling competition (Smiseth et al., 2007) and that inbred offspring beg less than outbred offspring (Chapter 4). In addition, eggs often hatch asynchronously leading to an age and size asymmetry between siblings (Rauter and Moore, 1999). Studies show that younger and smaller offspring beg more, but receive less provisioning than larger, older larvae (Andrews and Smiseth, 2013; Rauter and Moore, 1999; Smiseth and Moore, 2007). How competition and the allocation of resources by parents in mixed broods of inbred and outbred offspring are affected by inbreeding would provide information on another important social interaction within the family. Assuming that outbred offspring have a competitive advantage over inbred offspring, information on the begging of outbred offspring and the subsequent care parents provide would further test whether parents respond to the begging cues of offspring. Therefore, the effects of inbreeding on sibling competition would provide further evidence of the social costs of inbreeding on a family, in both offspring growth and survival, and in the care parents provide as a result.

7.1.4 Preferences For Outbred and Inbred Partners

I show that focal parents mated to an inbred partner provided more direct care than when mated to an outbred partner (Chapter 3). I also show a statistical interaction between the female and a males inbreeding status that affects the amount of care a female parent provides (Chapter 3). I suggest that future work investigates whether the effects of inbreeding mediate mate choice of an inbred or outbred partner. Inbred

partners may affect mating decisions of potential mates, based on the amount of care partners are expected to provide, which in turn is affected by their partner's inbreeding status. In the African cichlid fish, *Pelvicachromis taeniatus*, the amount of care each parent provides may affect the mating preferences of parents, in favour of related partners, and the authors argue that this is associated with the finding that related parents provide more cooperative care than unrelated parents (Thünken et al., 2007). The statistical interaction between male and female inbreeding status on the amount of direct care provided by females also predicts an interaction between male and female inbreeding status for mate preference, in which the focal individual's own inbreeding status may affect the assessment of a partner (Chapter 3). Several studies on invertebrates find that females prefer outbred males (Drayton et al., 2010; Ilmonen et al., 2009; Okada et al., 2011; Polkki et al., 2012). However, these studies do not investigate species that provide parental care, and so mating decisions are not predicted to be based on the later social consequences for the provision of care by outbred and inbred partners. The mating preferences for inbred and outbred partners would provide further information on how inbreeding affects mating decisions in relation to the amount of care each parent is expected to provide and how inbreeding affects the assessment of inbred and outbred partners.

7.2 Costs of Inbreeding in *N. vespilloides*

I show substantial costs of inbreeding in terms of offspring survival (Chapter 2), but I did not measure the subsequent fitness consequences for the social effects of inbreeding on parents and offspring. However, in *N. vespilloides* the costs of care are high (Cotter et al., 2010a; Ward et al., 2009), and I argue that the overcompensation of care when mating with an inbred partner (Chapter 3) incurs costs to the individual in terms of future reproduction and survival. Begging imposes costs on parents by potentially increasing the amount of care provided (Smiseth and Moore, 2002), whilst the social immunity and the amount of care parents provide to offspring determine the offspring's growth and survival (Arce et al., 2012; Eggert et al., 1998; Rozen et al., 2008). Hence, fitness costs to an individual are predicted to be associated with further costs to other individuals within a family. For example, increases in the provision of care when mated to an inbred partner or providing care to inbred offspring are predicted to reduce future reproductive success and survival of parents (Cotter et al., 2010a; Ward et al., 2009). Therefore, I suggest that increases in the amount of care as a result of inbreeding in other individuals incurs significant costs to parents and measuring the costs for the social effects of inbreeding in a family in terms of survival and future reproductive success to both offspring and parents would provide accurate measures of inbreeding costs to all members in a family.

7.2.1 Inbreeding Avoidance

I find no evidence of inbreeding avoidance by pre-copulatory mate choice when taking into account both mating strategies of *N. vespilloides* (Chapter 6). Males either search for a female in the absence of a carcass (Bartlett, 1987b; Eggert, 1992), or both males and females search for a carcass on which to reproduce (Eggert and Müller, 1997; Müller and Eggert, 1989; Scott, 1998). The direct and social effects of inbreeding in *N. vespilloides* are likely to impose costs within a family, and therefore it may be predicted that it is beneficial to avoid inbreeding. Inbreeding avoidance is not always predicted to evolve, even when the costs of inbreeding are high. Whether inbreeding avoidance occurs provides information on the selection pressures on inbred populations and so the occurrence of inbreeding in natural populations. The presence of inbreeding depression and the costs of avoiding inbreeding determines whether inbreeding avoidance evolves in a system (Waser et al., 1986). Inbreeding is expected to be tolerated when the costs of inbreeding avoidance are high, the loss of outbred matings are low and the more distantly related mates are optimal (Blouin and Blouin, 1988; Puurtinen, 2011). For example, in the linyphiid spider, *Oedothorax apicatus*, inbreeding depression imposes significant costs but there is no mating preference for non-siblings, suggesting that the likelihood of inbreeding is low (Bilde et al., 2007). The presence of inbreeding avoidance is difficult to generalise as it is closely associated with the population's history of inbreeding, its costs and prevalence (Bilde et al., 2007; Waser et al., 1986). In *N. vespilloides* I suggest that further mechanisms of inbreeding avoidance should be tested, such as post-copulatory mate choice. However, if inbreeding avoidance does not occur in this species, the high costs to a sub-social system suggest that the likelihood of inbreeding is low.

7.3 Implications For Parental Care

I found that partners provided more direct care when mated to an inbred parent, despite inbred parents not reducing the amount of direct care provided (Chapter 3) and that parents provided more care to inbred offspring that begged less (Chapter 4). My findings therefore provide evidence that parents cooperate over care using a 'sealed bid' (Houston and Davies, 1985), and that parents allocate care to offspring using honest signals from offspring (Godfray, 1991, 1995). I used inbreeding as a treatment that reduced an individual's fitness to interpret results concerning conflict resolution over the provisioning of care. However, I suggest that individuals did not directly assess the inbreeding status of the partner or offspring, but instead assessed or received additional signals or information associated with inbreeding. Given the level

of inbreeding in nature, it is unlikely that the mechanisms responsible for individuals to respond to inbred parents or offspring in their amount of care provided to evolve specifically to cope with inbreeding. Instead, I argue that when the behaviour or fitness of outbred individuals is altered due to interacting with inbred individuals, such effects will be mediated through mechanisms that evolved to serve an adaptive function in other contexts. Considering the use of inbreeding to test pre-existing mechanisms, it is a valuable treatment in respect to parental care theory because it reduces the fitness of an individual at the genomic level, affecting a wide range of metabolic and behavioural genes (Ayroles et al., 2009; Kristensen et al., 2006). This is useful because many studies only test the effect of quality of partners by reducing productivity by wing clipping, adding weights, or in offspring by varying hunger levels (Harrison et al., 2009; Hinde and Kilner, 2007; Rauter and Moore, 2004; Smiseth et al., 2005; Suzuki and Nagano, 2009). However, these either produce short-term reductions in quality or adjust the behaviour of the individual, whilst inbreeding subjects the individual to long-term reductions in fitness throughout its life. In previous studies in *Nicrophorus*, compensation has only been investigated in response to the removal of a partner or to the reduction of care by adding weights, not taking account of whether the focal individual assesses the condition of the partner, rather than the care provided (Smiseth et al., 2005; Suzuki and Nagano, 2009). Hence, I propose that the reduction in fitness as a consequence of inbreeding throughout an inbred individual's life represents a treatment that reduces fitness at the genomic level, rather than other short-term manipulations which do so at a behavioural or physiological level. The evidence on biparental cooperation and parent-offspring communication (Chapter 3 and 4) and previous mate manipulation experiments (Suzuki and Nagano, 2009) suggests that this may be an important distinction when empirically testing predictions on partner responses from parental care theory.

7.4 Implications For Inbred Populations

I find evidence of strong direct, intergenerational and social effects of inbreeding on parent and offspring traits in a species with elaborate biparental care. To reliably estimate inbreeding depression in wild populations, I suggest that studies account for the social effects of inbreeding by extending fitness measures to individuals that have fitness-related social interactions with inbred individuals. These social effects of inbreeding within a family emphasise the need to accurately estimate the costs of inbreeding by accounting for all social interactions between parents and offspring and other social traits, rather than only accounting for inbred individuals. Future studies on species with parental care should consider the effect parents and offspring have on one another: whether parents provide more variable care, whether offspring demand or

require more care from parents, whether parents suffer if a partner is inbred and whether any effects of inbreeding are increased in harsher social environments. Such effects may further increase or reduce the costs of inbreeding, to which small populations are at particular risk, and inbreeding depression that is underestimated puts such populations at a higher extinction risk (Haikola, 2003; Hedrick and Kalinowski, 2000). Here, I focussed on traits associated with parental care, however other social traits with strong fitness associations should also be considered. Parental care and begging between parents and offspring in a family, within which all individuals can be assumed to be a minor form of a society, may be treated as any other kin-selected social behaviour (Cotter and Kilner, 2010a). Therefore, the effects of inbreeding on cooperation and parent-offspring communication may also be applicable to extra-familial social interactions such as mating, competition and other forms of cooperation that experience evolutionary conflict or are under strong selection.

7.5 Concluding Remarks

Evidence from previous studies show that inbreeding in an individual can influence the fitness of others, in particular offspring or mating partners. Here, I present evidence of strong social effects of inbreeding on the interactions between two individuals, in which the effects of inbreeding were complex and largely unpredicted. I suggest that measuring inbreeding in one individual simplifies the effects of inbreeding, and in species that provide parental care or rely on other social interactions, inbreeding may have subtle and complex effects on how individuals within an inbred population interact and affect one another's fitness.

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Intergenerational effects of inbreeding in *Nicrophorus vespilloides*: offspring suffer fitness costs when either they or their parents are inbred

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offspring survival;
parental care.

Abstract

Inbreeding depression is the reduction in fitness caused by mating between related individuals. Inbreeding is expected to cause a reduction in offspring fitness when the offspring themselves are inbred, but outbred individuals may also suffer a reduction in fitness when they depend on care from inbred parents. At present, little is known about the significance of such intergenerational effects of inbreeding. Here, we report two experiments on the burying beetle *Nicrophorus vespilloides*, an insect with elaborate parental care, in which we investigated inbreeding depression in offspring when either the offspring themselves or their parents were inbred. We found substantial inbreeding depression when offspring were inbred, including reductions in hatching success of inbred eggs and survival of inbred offspring. We also found substantial inbreeding depression when parents were inbred, including reductions in hatching success of eggs produced by inbred parents and survival of outbred offspring that received care from inbred parents. Our results suggest that intergenerational effects of inbreeding can have substantial fitness costs to offspring, and that future studies need to incorporate such costs to obtain accurate estimates of inbreeding depression.

Introduction

Inbreeding depression is the reduction in fitness of individuals resulting from matings between related mates, and is caused mainly by the expression of deleterious recessive alleles due to an increase in homozygosity (Falconer & Mackay, 1996; Lynch & Walsh, 1998; Charlesworth & Willis, 2009). The study of inbreeding depression is an important issue in evolutionary and conservation biology because it leads to selective pressures that shape mating systems and behaviours (Charlesworth & Willis, 2009), and because it poses a conservation concern to many wild populations (Ralls *et al.*, 1979; Crnokrak & Roff, 1999; Keller & Waller, 2002; Armbruster & Reed, 2005). There is ample evidence for significant costs of inbreeding from studies on a wide range of taxa, including birds, mammals, insects and plants. These studies show that the effects of inbreeding may have detrimental effects on a wide range of juvenile and adult traits, including hatching success, offspring survival and the number of eggs laid by adult females (Noordwijk & Scharloo, 1981; Keller, 1998; Slate *et al.*, 2000; Szulkin *et al.*, 2007). Due to these wide-ranging effects, accurate estimates of inbreeding need to be based on fitness consequences measured across the entire life cycle of an individual (Charlesworth & Charlesworth, 1987). The finding that inbreeding affects adult traits suggests that inbreeding in parents also could affect the fitness of outbred offspring. If so, accurate estimates of inbreeding depression need to account for intergenerational effects of inbreeding in parents on offspring fitness. Unfortunately, there has so far been little attention to the potential significance of intergenerational effects of inbreeding in species where offspring fitness is determined or influenced by their parents.

Current studies on intergenerational effects of inbreeding have focused mainly on plants, and have shown that the inbreeding coefficients of parents

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influence fitness traits of progeny (del Castillo, 1998; Riginos *et al.*, 2007). There are far fewer studies of intergenerational effects in inbreeding in animals, where the potential for such effects may be particularly important because many species have elaborate parental care (Clutton-Brock, 1991; Royle *et al.*, 2012). For example, in oldfield mice, inbreeding in parents led to a reduction in male (but not female) parental care, although inbreeding in parents had no effect on offspring fitness (Margulis, 1998). A recent study on red deer, *Cervus elaphus*, which compared the effects of inbreeding in offspring traits when either the offspring themselves or the female parents were inbred, found that inbreeding in offspring was associated with a reduction in birthweight and first-year survival, while the inbreeding in female parents had no effects on the same offspring traits. Thus, this study suggests that intergenerational effects of inbreeding are weak compared with the direct effects of inbreeding (Walling *et al.*, 2011). On the other hand, there is support for intergenerational effects of inbreeding from a study on song sparrows, which found that offspring of inbred female parents had a lower immune response than offspring of outbred parents (Reid *et al.*, 2003). Furthermore, in great tits, *Parus major*, offspring of inbred parents have lower fledging success, recruitment into the breeding population and lower reproductive success than offspring of outbred parents (Szulkin *et al.*, 2007). These findings suggest that there might be intergenerational effects of inbreeding resulting from detrimental effects of inbreeding on the amount of parental care provided to offspring.

To improve our understanding of the potential significance of intergenerational effects of inbreeding, it is now important to investigate inbreeding depression in offspring traits both when the offspring themselves are inbred and when the parents are inbred. Here, we report the results of experiments on the burying beetle *Nicrophorus vespilloides*; an insect with highly elaborate parental care, whereby one or both parents defend the carcass and the brood against conspecific competitors and microbial decomposers, and provide predigested carrion to the begging larvae (Eggert *et al.*, 1998; Smiseth *et al.*, 2003; Rozen *et al.*, 2008; Arce *et al.*, 2012). Parental care has a strong effect on offspring fitness by enhancing offspring survival and growth and speeding up the offspring's rate of development (Eggert *et al.*, 1998; Smiseth *et al.*, 2003). We conducted two controlled experiments where the inbreeding status of either the offspring or the parents were manipulated through experimental breeding, allowing us to estimate the effects of inbreeding on offspring traits when either the offspring themselves were inbred or when their parents were inbred. We monitored effects of inbreeding in offspring and parents on offspring traits during different stages of the life cycle, including hatching success of eggs, survival and growth of larvae during the period when they received care from their parents, and after independence. In the experiment on intergenerational effects of inbreeding, we manipulated inbreeding in male and female parents to test for potential sex differences in the effects of inbreeding in parents on offspring fitness.

Materials and methods

Study species

Like all members of the genus *Nicrophorus*, *N. vespilloides* breeds on carcasses of small vertebrates, which are buried underground, rolled into a ball and then prepared and maintained by both parents (Eggert & Muller, 1997; Scott, 1998). Females lay eggs in the soil around the carcass over a mean period of 30 h and the eggs hatch about 60 h after laying (Smiseth *et al.*, 2006). Once the larvae hatch, they crawl to the carcass, where they obtain food by self-feeding directly from the carcass and by begging for regurgitated food from the parents (Smiseth *et al.*, 2003). Parents provide elaborate care that includes provisioning of food to the larvae, protecting and defending the brood from predators and competitors and applying antimicrobials to defend the brood from microbes (Eggert *et al.*, 1998; Smiseth *et al.*, 2003; Rozen *et al.*, 2008; Arce *et al.*, 2012). Females are more involved in direct care of the larvae than males, and also provide care for a longer period of time (Smiseth & Moore, 2002; Eggert *et al.*, 2008). The larvae disperse from the carcass about 5–6 days after hatching, pupate about 10–12 days after dispersal and finally eclose as adults about 12 days after pupation.

General methodology

We used beetles from a large outbred laboratory population maintained at The University of Edinburgh. Inbreeding in the stock population was avoided by maintaining a large stock population and ensuring that breeding occurred among unrelated or distantly related individuals, defined as when two individuals did not share a common ancestor two generations or more back in time. The population comprised of fifth-generation beetles from lines originally collected at Corstorphine Hill, Edinburgh, and 16th-generation beetles from lines originally collected at Kennel Vale, Cornwall. Thus, we are confident that the stock population would not have been subject to any significant forms of inbreeding, purging or selection that would have biased the estimates of inbreeding depression in our experiments.

The beetles were maintained at 20 °C under constant light conditions in transparent plastic containers (12 cm × 8 cm and 2-cm high). Nonbreeding adults were fed organic beef twice a week. We only used virgin beetles for the experiments, and each individual was only mated once. Experimental pairs were bred in

transparent plastic boxes (17 cm × 12 cm and 6-cm high) by providing them with 1 cm of moist soil and a previously frozen mouse carcass with a mass of 20 ± 5g. The mice were supplied from Livefoods Direct Ltd, Sheffield, UK. The containers were checked for eggs on days 1–7 after pairing by noting the number of laid eggs and the number of unhatched eggs (i.e. decomposing eggs) that were visible through the base and sides of the transparent container. We used this method as it reliably estimates the number of eggs laid while at the same time reducing the risks of damaging eggs through handling, which might reduce hatching success (Monteith *et al.*, 2012). The containers were checked for dispersing larvae on days 10–12 after pairing by noting whether the larvae had left the carcass. Once the larvae had dispersed, we recorded the date, the number of larvae that were alive and the mass of the brood. The dispersed larvae were then placed in individual containers and left to pupate and eclose. The individual containers were again checked for eclosing adults 27–34 days after pairing.

Experimental design

Experiment 1: Inbreeding in offspring

To examine the effects of inbreeding when the offspring themselves were inbred, we used a block design similar to the one used by Fox *et al.* (2008). Each block comprised of eight beetles, four male and four female, derived from two unrelated families of the stock population (i.e. families that did not share a common grandparent). Each block produced two broods of inbred offspring by mating a female from each of the two families with her brother (i.e. full-sibling mating), and two broods of outbred offspring by mating a female from each of the two families with the unrelated male from the other family (Fig. 1a). Such a block design provides a powerful means for attributing variation in offspring trait values to inbreeding among offspring in a way that controls for potential confounding effects due to genetic differences among the different families used in the experiment (Fox *et al.*, 2008). Controlling for genetic differences among families is highly advantageous in studies of inbreeding depression because families share genes that may affect the mean values of the traits that are potentially affected by inbreeding (Fox, 2005). Thus, the use of a blocked design ensures that the same set of alleles contributes to both inbred and outbred broods, and potential effects due to genetic differences among families can therefore be excluded (Fox, 2005). This design also ensures that all parents used in the experiment are outbred, such that any effect due to inbreeding would be caused by inbreeding among the offspring.

We set up 15 blocks in this experiment, consisting of 10 families of 16th generation and 13 families of fifth generation beetles, yielding a total of 30 inbred broods and 30 outbred broods. Three inbred matings (in three separate blocks) were excluded because one of the parents died prior to egg laying. We removed all males from the breeding container 7 days after pairing because this experiment was designed to test for effects of inbreeding in the offspring and their subsequent fitness. Previous studies show that that the extent to which males stay with the brood to provide care or desert is highly variable in this species (Smiseth & Moore, 2002), and that the removal of the male has no effect on offspring fitness (Smiseth *et al.*, 2005). Thus, the male was removed to control for potential variation in male behaviour.

We measured effects of inbreeding in the offspring on the following traits: *(i) Number of eggs* recorded as

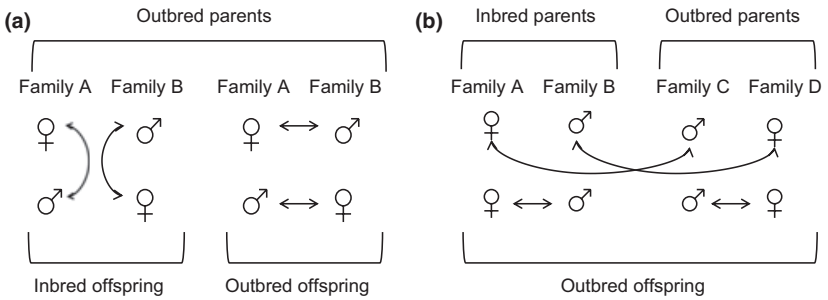

Fig. 1 The two block designs used to test for the effects of inbreeding in offspring and parents in *Nicrophorus vespilloides*. (a) In Experiment 1, each block comprised four males and four females derived from two unrelated families of the stock population. Each block produced two broods of inbred offspring by mating a female from each of the two families with her brother (i.e. full-sibling mating), and two broods of outbred offspring by mating a female from each of the two families with the unrelated male from the other family. (b) In Experiment 2, each block comprised four males (two outbred and two inbred) and four females (two outbred and two inbred) derived from four unrelated families. Mating within each block ensured that all offspring were outbred, and that these offspring were produced and cared for by one of the following four treatment combinations: an inbred female mated to an inbred male; an inbred female mated to an outbred male; an outbred female mated to an inbred male; and an outbred female mated to an outbred male.

the number of eggs laid for each brood; *(ii) Hatching success* recorded by counting the number of unhatched eggs (i.e. decomposing eggs) visible through the base and sides of the container, subtracting this number from the total number of eggs laid to give the number of successfully hatching eggs, and then calculated relative to the total number of eggs laid; *(iii) Time to dispersal* recorded as the number of days from when the parents were paired up to when larvae dispersed from the carcass; *(iv) Survival to dispersal* recorded by counting the number of larvae dispersing from the carcass, and then calculated relative to the total number of eggs laid; *(v) Size at dispersal* recorded by weighing the whole brood to the nearest 0.1 mg, and dividing the brood mass by the number of larvae in the brood to yield a mean body mass per larva in the brood; *(vi) Overall offspring survival* recorded as the number of larvae surviving to dispersal from the carcass, and then calculated relative to the number of eggs laid to for a given brood; *(vii) Survival to eclosion* recorded as the number of larvae successfully eclosing relative to the number of larvae dispersing from the carcass for a given brood. For measures of survival (hatching success, survival to dispersal, overall offspring survival and survival to eclosion), we calculated mean values for each treatment based on the proportion of surviving offspring for each brood, while we used the number of surviving and nonsurviving offspring as binomial variables in our statistical models (see below).

Experiment 2: Inbreeding in parents

To test for possible intergenerational effects of inbreeding in parents on the offspring's fitness, we used a novel block design based on the inbred and outbred individuals generated from Experiment 1. The design comprised of eight beetles derived from four unrelated families (i.e. families that did not share a common grandparent). Each block comprised two females from two different families (i.e. two sets of full-sibling sisters), one set of which was inbred and one of which was outbred, and two males from two additional families (i.e. two sets of full-sibling brothers), one of which was inbred and one of which was outbred (Fig. 1b). These beetles were mated to produce the following four treatments: (i) an inbred female mated to an inbred male; (ii) an inbred female mated to an outbred male; (iii) an outbred female mated to an inbred male; (iv) an outbred female mated to an outbred male (Fig. 1b). Thus, this set-up produced a two-by-two factorial design where parents of either sex were either inbred or outbred. The design allowed us to compare the effects of inbreeding in male and female parents on the offspring's fitness, while at the same time ensuring that all offspring were outbred (offspring were always outbred because all parents, inbred or outbred, were mated to an unrelated male or female; Falconer & Mackay, 1996; Frankham *et al.*, 2002). Thus, any effects of inbreeding on offspring fitness in this experiment would be due to inbreeding among parents. Furthermore, this factorial design also allowed us to detect any potential differences in the effects of inbreeding in females and males. Such effects might be found in this species because female parents are more involved in direct care for the larvae than males (Eggert & Muller, 1997; Smiseth & Moore, 2002). Because we were interested in testing for such a sex difference, we did not remove the male from the breeding container in this experiment, thus allowing both parents to provide parental care. We created 11 blocks, yielding a total of 11 broods of each treatment with the exception of the treatment comprising an inbred female and an outbred male, which was reduced to 10 broods because one adult died before mating. To determine whether inbreeding in parents had an effect on offspring traits, we estimated the same offspring and adult traits as in Experiment 1 (with the exception of time to dispersal and survival to eclosion, which were not recorded due to time limitations), following the same procedures as described above for Experiment 1.

Statistical analysis

All data was analysed using R version 2.15. We used linear mixed-effects models for traits that had a normal error distribution (the number of eggs laid and size at dispersal in both experiments), and we used generalized linear mixed models for traits with a Poisson error structure (time to dispersal), and for traits with a binomial error structure (hatching success, survival to dispersal, overall offspring survival and survival to eclosion). All analyses were based on family means (Fox *et al.*, 2008). In Experiment 1, the experimental treatment (i.e. whether offspring were outbred or inbred) was assigned as a fixed effect, whereas in Experiment 2, the experimental treatments (i.e. whether males or females were outbred or inbred) were added as two fixed effects (i.e. one for each sex). In all models, block was assigned as a random factor. In the models of three traits (size at dispersal, survival to dispersal and survival to eclosion), we included additional covariates in the initial statistical models because these covariates were predicted to have an effect on the trait in question. First, in the model of size at dispersal, we included brood size as an additional fixed effect because brood size is likely to determine the amount of resources available to each offspring and therefore the size of offspring (Smiseth *et al.*, 2007). Second, in the model of survival to dispersal, we included mouse mass as an additional fixed effect because survival may be influenced by the amount of resources available to the brood. Third, in the model of survival to eclosion, we added size at dispersal as an additional fixed effect because the size of the larvae may have a possible effect on the larvae's ability to eclose successfully. We based

decisions as to whether to include these covariates in the final model based on AIC model-selection criteria, according to which a covariate should be added if it significantly improves the fit of the model. In the analysis on survival to dispersal, which aimed to establish the effect of inbreeding on larval survival from hatching to the time of dispersal, we excluded two broods from Experiment 2 where no eggs hatched. In the analyses on inbreeding in parents (Experiment 2), we always first tested for a main effect of inbreeding depending on the sex of the parent before we generated separate models for the interaction between the inbreeding status of males and females. For all traits, we calculated inbreeding depression as a proportional change in mean fitness of inbred (w_i) and outbred (w_o) individuals using the equation as $\delta = w_o - w_i/w_o$ (Hedrick & Kalinowski, 2000). We note that the tables summarize the effect of inbreeding on each trait while controlling for the effects of block and other additional fixed effects listed above, while the figures show means and standard errors for each trait based on the raw data. Whenever there are apparent discrepancies between tables and figures, we always base inferences about the effects of inbreeding on the statistical analyses reported in the tables.

Table 1 Direct effects of inbreeding on offspring traits in *Nicrophorus vespilloides*. For each trait, there is information on parameter estimates (Par) with standard error (SE), test statistics (*t*-value for number of eggs and size at dispersal, and Z-value for time to dispersal, hatching success, survival to dispersal, overall offspring survival and survival to eclosion) and *P* values based on comparisons between outbred and inbred offspring. All data are provided by linear mixed-effects models of egg number and size at dispersal and generalized linear mixed models for time to dispersal (fitted with Poisson error structure), hatching success, survival to dispersal, overall offspring survival and survival to eclosion (fitted with a binomial error structure), in which experimental block is assigned as a random factor in all cases.

Trait	Par	SE	t/Z value	*P* value
Number of eggs	2.83	2.1	1.35	0.184
Hatching success (%)	−0.821	0.22	−3.83	0.0001
Time to dispersal (days)	0.014	0.081	0.18	0.855
Survival to dispersal (%)	−0.43	0.126	−3.42	0.0006
Size at dispersal (g)	−0.004	0.008	−0.462	0.647
Overall offspring survival (%)	−0.535	0.116	−4.611	< 0.0001
Survival to eclosion (%)	−0.733	0.18	−4.12	< 0.0001

Results

Experiment 1: Inbreeding in offspring

We first examined the additional covariates in our initial models to determine whether to include these in the final models for the effects of inbreeding on size at dispersal, survival to dispersal and survival to eclosion. Although larger broods produced larvae that were significantly smaller at dispersal ($t_{40} = -5.491$, $P < 0.001$), AIC model selection suggested that brood size should not be included in the final model for size at dispersal. Given that these results indicate some ambiguity concerning the effect of brood size, we conducted two separate tests in which brood size was either included or excluded. These tests confirmed that the exclusion or inclusion of brood size had no effect on estimates of inbreeding depression, and we therefore report effects from a model in which brood size was excluded. Mouse mass had no significant effect on larval survival to dispersal (Z = 0.3, $P = 0.76$), and was not included in the final model on survival to dispersal based on AIC model selection. Finally, larvae that were larger at dispersal were more likely to survive to eclosion (Z = −2.826, $P = 0.005$), and size at dispersal was included in the final model for survival to eclosion based on AIC model selection.

There was no evidence that females mated to a full-sib male laid a different number of eggs than females mated to an unrelated male (Table 1; Fig. 2). Thus, there was no evidence suggesting that females adjusted their reproductive investment in response to the risk of inbreeding in their offspring.

Inbred offspring suffered substantial inbreeding depression in a wide range of traits (Table 1, Fig. 2), including hatching success of eggs (reduced by 3.5%), survival to dispersal (reduced by 8.5%), overall offspring survival from hatching to dispersal (reduced by 11.2%) and survival to eclosion as adults (reduced by 8.7%; Table 3). Inbred offspring suffered a significant reduction in survival both during the period when larvae depend on parental care (i.e. from egg laying to dispersal from the carcass) and after independence (from dispersal to eclosion; Table 1). In contrast, there was no evidence of a difference between inbred and outbred offspring with respect to the time to dispersal and size at dispersal (Table 1), suggesting that inbreeding had no detectable effect on the duration of life-history stages or the body size of surviving offspring.

Experiment 2: Inbreeding in parents

At first, we examined the additional covariates in our initial models to determine whether these should be included in the final models on the effects of inbreeding on the size at dispersal and survival to dispersal. As in Experiment 1, larger broods produced significantly smaller larvae at dispersal ($t_{17} = -3.97$=, $P = 0.001$), but in this case, brood size was included in the final model based on AIC model selection. Mouse mass had no significant effect on the survival to dispersal (Z = −1.338, $P = 0.181$) and was not included in the final model of survival to dispersal based on AIC model selection.

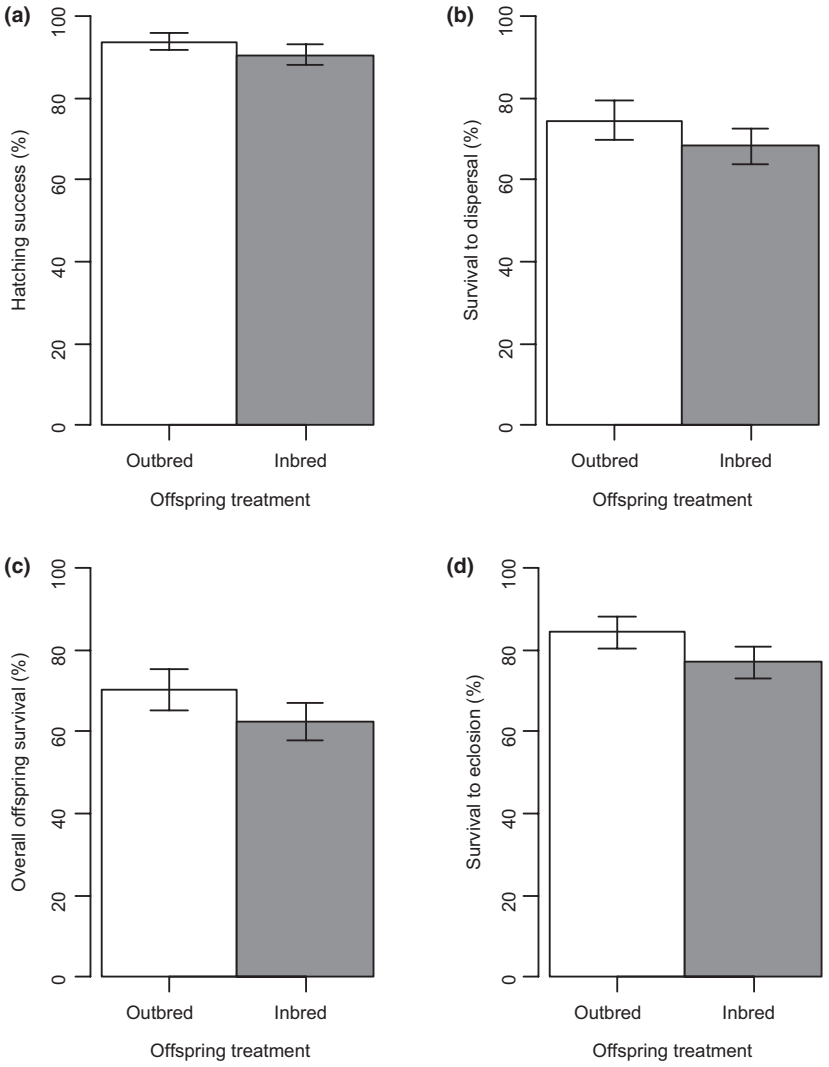

Fig. 2 Direct effects of inbreeding on offspring traits in *Nicrophorus vespilloides*. Comparisons of hatching success (a), survival to dispersal (b), overall offspring survival (c) and survival to eclosion (d) when offspring were outbred (white bars) or inbred (grey bars) (mean ± SE).

Outbred offspring suffered significant costs due to inbreeding in their male and/or female parents, causing a reduction in hatching success of eggs, survival to dispersal and overall offspring survival (Table 2). Our results indicate that the intergenerational effects of inbreeding in parents on offspring traits were often complex, involving in some cases, sex differences in the effects of inbreeding, and in other cases, interaction effects due to inbreeding in both sexes (Table 3). There was no evidence that inbred and outbred females laid a different number of eggs as indicated by the nonsignificant main effect of inbreeding in females. However, females laid significantly more eggs when mated to an inbred male as compared with when mated to one that was outbred (Table 2). The main effect of inbreeding in males was associated with a significant effect of the interaction between inbreeding in males and females (Table 3; Fig. 3a). Inbred females increased the number of eggs they laid by 70% when they were mated to an inbred male as opposed to when they were mated to an outbred male. In contrast, outbred females laid a similar number of eggs regardless of whether they were mated to an inbred or an outbred male.

There was no evidence that inbreeding in either male or female parents affected the hatching success of outbred eggs (Table 2). However, hatching success was significantly influenced by the interaction between inbreeding in male and female parents. Hatching success of eggs sired by inbred males was reduced by 12% when he was mated to an inbred female as opposed to when he was mated to an outbred female. In contrast, hatching success of eggs sired by outbred males appeared similar, regardless of whether they were mated to an inbred or an outbred female (Table 3). Inbreeding in female parents caused a significant reduc-

Table 2 Intergenerational effects of inbreeding in parents on the traits of outbred offspring in *Nicrophorus vespilloides*. For each trait, there is information on parameter estimates (Par) with standard error (SE), test statistics (*t*-value for number of eggs and size at dispersal, and Z-value for hatching success, survival to dispersal and overall offspring survival) and *P* values based on comparisons of outbred offspring produced by and receiving care from males that were outbred or inbred, females that were outbred or inbred and the effect of the interaction between inbreeding in males and females. All data are provided by linear mixed-effects models of egg number and size at dispersal, and generalized linear mixed effects models of hatching success, survival to dispersal and overall offspring survival (fitted with a binomial error structure), in which experimental block is assigned as a random factor in all cases.

	Male				Female				Interaction			
Trait	Par	SE	t/Z value	*P* value	Par	SE	t/Z value	*P* value	Par	SE	t/Z value	*P* value
Number of eggs	7.09	2.66	2.66	0.01	−0.64	2.66	−0.239	0.812	11.93	5.04	2.37	0.0248
Hatching success (%)	−0.304	0.16	−1.881	0.06	−0.17	0.16	−1.069	0.285	−1.51	0.34	−4.431	< 0.0001
Survival to dispersal (%)	−0.097	0.16	−0.61	0.541	−0.407	0.15	−2.67	0.0076	1.0	0.32	3.14	0.0017
Size at dispersal (g)	0.007	0.01	0.822	0.422	0.002	0.01	0.268	0.792	0.011	0.02	0.676	0.508
Overall offspring survival (%)	−0.219	0.13	−1.639	0.101	−0.311	0.13	−2.35	0.0187	0.088	0.27	0.328	0.743

Table 3 Inbreeding depression (δ) was calculated for all traits as $\delta = w_o - w_i / w_o$ such that trait values associated with an inbred individual is compared to that of an outbred individual. Offspring δ was estimated for the direct effects of inbreeding when the offspring themselves were inbred using data from Experiment 1. Male δ, female δ and both parents δ were estimated as intergenerational effects in outbred offspring due to effects of inbreeding in male parents, female parents and both parents using data from Experiment 2. Male δ was calculated separately for males mated to inbred and outbred females; that is, the column Male parent δ (inbred female) reports the reduction in offspring fitness due to inbreeding in males when males were mated to an inbred female. Likewise, female δ was calculated separately when females were mated to inbred and outbred males. Both parents δ was calculated by comparing trait values of the treatment in which both parents were inbred with the trait values when both the parents were outbred.

		Male parent δ		Female parent δ		
Trait	Offspring δ	Female outbred	Female inbred	Male outbred	Male inbred	Both parents δ
Number of eggs	−0.092	−0.049	−0.702	0.264	−0.193	−0.252
Hatching success (%)	0.035	−0.067	0.022	0.036	0.117	0.057
Time to dispersal (days)	−0.015	–	–	–	–	–
Survival to dispersal (%)	0.085	0.236	−0.218	0.386	0.022	0.253
Size at dispersal (g)	0.018	−0.035	−0.084	0.035	−0.011	−0.047
Overall offspring survival (%)	0.112	0.138	−0.3	0.4	0.095	0.221
Survival to eclosion (%)	0.087	–	–	–	–	–

tion in larval survival to dispersal, while there was no evidence that inbreeding in male parents had an effect on larval survival to dispersal (Table 2, Table 3). The main effect of inbreeding in females was associated with a significant effect of the interaction between inbreeding in males and females (Table 3). Inbreeding in females reduced larval survival to dispersal by 38% when females were mated to an outbred male, whereas there was no effect of inbreeding in females when females were mated to an inbred male (Table 3). Inbreeding in female parents reduced overall offspring survival by 40%, while inbreeding in male parents had no such effect (Fig 3; Table 3). However, there was no significant effect of the interaction between inbreeding in male and female parents on overall offspring survival (Table 2). There was no evidence that inbreeding in males and females, or the interaction between the two, caused a reduction in the size of larvae at dispersal (Table 2).

Discussion

Here, we report evidence from an experimental study on the burying beetle *N. vespilloides* showing that inbred offspring suffer significant fitness costs and that outbred offspring suffer significant fitness costs when their parents are inbred. Our results provide a valuable contribution to the understanding of inbreeding depression by demonstrating that offspring suffer the costs of inbreeding not only when they themselves are inbred but also when they are produced by and receive care from parents that are inbred. We argue that such intergenerational effects of inbreeding can be important in many species, particularly in those where parents provide elaborate care for their offspring as in birds and mammals, and we suggest that further work on inbreeding in these species need to consider the potential implications of intergenerational effects of inbreeding. On the basis of our results, we propose that the

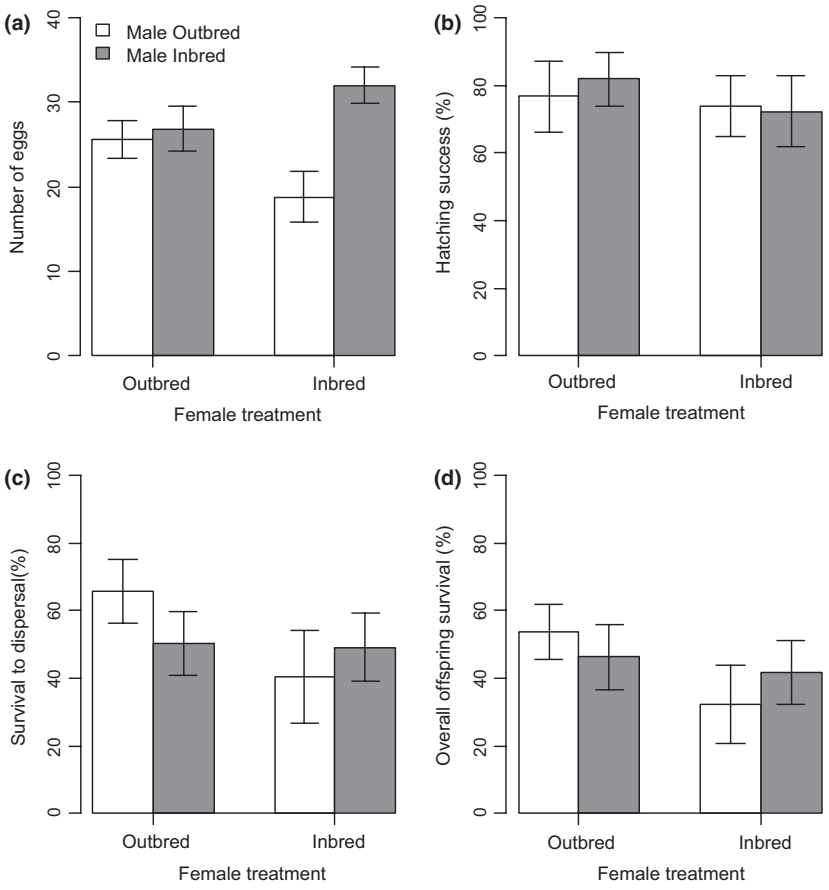

Fig. 3 Intergenerational effects of inbreeding in parents on the traits of outbred offspring in *Nicrophorus vespilloides*. Comparison of the number of eggs laid (a), hatching success (b), survival to dispersal (c) and overall offspring survival (d) when outbred offspring were produced by and received care from outbred or inbred female parents (female treatment) and outbred (white bars) or inbred (grey bars) male parents (mean ± SE).

costs of inbreeding might extend to individuals other than those that are inbred, and that such indirect effects of inbreeding may be important in a wide range of social contexts in which the fitness of an outbred individual depends on the behaviour of individuals that are inbred. Below, we provide a more detailed discussion of the major implications of our results.

The main aim of this study was to examine both direct effects of inbreeding when the offspring themselves were inbred, and intergenerational effects of inbreeding when outbred offspring were produced by and received care from inbred parents. Direct effects of inbreeding were associated with a reduction in overall offspring survival by 11%, whereas intergenerational effects of inbreeding were associated with a reduction in overall offspring survival by 40% when females were inbred (and males were outbred; Table 3). In Experiment 1, males were removed from the brood to remove potential effects due to variation in male desertion, while males were left with the brood in Experiment 2 to provide estimates on the relative importance of inbreeding in males and females. Because of this difference in the design between the two experiments, caution is required when comparing the estimates of inbreeding depression when the offspring themselves and their parents were inbred. For example, leaving males with the brood in Experiment 2 may have influenced offspring fitness either because males would have had the opportunity to provide additional care or because deserting males could have cannibalized some larvae (Scott & Gladstein, 1993). Nevertheless, our results suggest that intergenerational effects of inbreeding may be comparable in strength, or even stronger than the direct effects of inbreeding in *N. vespilloides*. This finding contrasts with a recent study on red deer, suggesting that the direct effects of inbreeding are much stronger than any intergenerational effects of inbreeding (Walling *et al.*, 2011).

The finding that inbreeding had severe fitness costs to offspring both when they themselves were inbred and when the parents were inbred is somewhat surprising because these effects must be based on different mechanisms. Although we did not specifically set out to investigate the mechanisms behind these effects, our results provide some suggestions for possible mechanisms based on observed reductions in survival during specific developmental stages. The observed reduction in hatching success and offspring mortality when the

offspring (eggs or larvae) were inbred is likely to reflect mechanisms taking place after fertilization, such as an increase in embryonic or larval mortality directly caused by the inheritance of rare homozygous deleterious recessive alleles. However, this mechanism cannot explain the reduction in hatching success or offspring survival when parents were inbred, because in this situation, the offspring (eggs or larvae) were outbred. Instead, the reduction in hatching success when parents were inbred must reflect mechanisms taking place prior to or during fertilization, such as reduced quality or viability of eggs and sperm. Similar results have been reported in a recent study on the seed beetle, *Stator limbatus*, in which eggs laid by inbred females were less likely to hatch than those laid by outbred females (Messina *et al.*, 2013). Finally, we note that the reduction in offspring survival from hatching to dispersal when parents were inbred is likely to reflect mechanisms taking place after hatching, such as reduced quantity or quality of care provided by inbred parents.

We found some evidence for a sex difference in the intergenerational effects of inbreeding. First, inbreeding in males was associated with an increase in the number of eggs laid by an inbred female, whereas inbreeding in females had no such effect. Although this finding is consistent with females attempting to compensate for an expected reduction in hatching success when mated to an inbred male, we believe that such a conclusion is not warranted because we have not been able to replicate this finding in later experiments based on the same design (S.N. Mattey & P.T. Smiseth, unpublished data). Second, inbreeding in females was associated with a reduction in larval survival from hatching to dispersal and overall offspring survival, whereas inbreeding in males had no such effect, a finding that we have replicated in later experiments (S.N. Mattey & P.T. Smiseth, unpublished data). These results are consistent with females being more involved in provisioning of food to larvae than males in *N. vespilloides* (Smiseth & Moore, 2002; Smiseth *et al.*, 2005). Thus, the finding that inbreeding in females may have had a stronger impact on larval survival from hatching to dispersal may simply reflect that females are more involved in the form of care that is likely to have the strongest impact on larval survival; that is, the provisioning of food to larvae (Eggert *et al.*, 1998; Smiseth *et al.*, 2003). The finding that inbreeding in males had no detectable effect on larval survival from hatching to dispersal suggests that inbreeding had no effect on how males left with the brood influenced their offspring's fitness via parental care or cannibalism.

We also found evidence for significant effects of the interaction between inbreeding in males and females with respect to the number of eggs laid, hatching success of eggs and larval survival from hatching to dispersal. These interaction effects suggest that the intergenerational effects of inbreeding in a parent of a given sex depend not only on its own inbreeding status but also on that of its partner. These interaction effects are likely to depend on different mechanisms. For example, the interaction effect on hatching success may reflect that successful fertilization depends on the quality of both sperm and eggs (both of which may be reduced by inbreeding), although further work is needed to confirm whether this is indeed the case. In contrast, the interaction effect on larval survival from hatching to dispersal occurs during the period where larvae receive care from their parents, and may potentially reflect that inbreeding alters the behavioural dynamics between male and female parents. Such effects might be expected in species with biparental care because each parent often adjusts its contribution towards care based on the amount of care provided by its partner (Houston & McNamara, 2005; Lessells & McNamara, 2012). These considerations suggest that intergenerational effects of inbreeding may alter the complex behavioural dynamics of interactions among family members, and there is now a need for further work to investigate this intriguing possibility.

Although the finding that outbred offspring suffered from reduced survival when cared for by inbred parents suggests that inbred parents provide less care than outbred parents, it is important to recognize that other mechanisms such as reductions in maternal immunity cannot be excluded (Reid *et al.*, 2007). Nevertheless, our study provides some indications that inbreeding may affect the amount of care provided by parents. First, inbreeding in parents had the strongest effect on offspring fitness during the period from hatching to dispersal. This represents the stage in the offspring's life cycle during which parents provide care for offspring (Eggert *et al.*, 1998; Smiseth *et al.*, 2003). Second, inbreeding in female parents was more detrimental for survival to dispersal than inbreeding in male parents, which is consistent with females being more engaged in direct parental care for larvae than males in this species (Smiseth & Moore, 2002). At present, surprisingly few studies have documented effects of inbreeding on parental care. A notable exception is a study on oldfield mice, which showed that inbreeding led to a reduction in the amount of care provided by males (but not by females), although in this case, inbreeding in parents had no effect on offspring survival (Margulis, 1998). Further work on a wide range of taxa is now needed to examine the potential effects of inbreeding on parental care.

The finding that intergenerational effects of inbreeding in parents can have strong and detrimental effects on the offspring's fitness has important implications for the study of inbreeding. First, if intergenerational costs of inbreeding are commonplace, ignoring such costs runs the risk of underestimating the true cost of inbreeding. This issue might be particularly problematic when attempting to estimate inbreeding depression in species with elaborate forms of parental care, such as birds and

mammals. Second, intergenerational effects of inbreeding may be part of a larger class of inbreeding effects that occur in a wide range of social contexts. These contexts include all instances where the fitness of an outbred individual depends on behavioural interactions with others and where inbreeding influences the behaviour of the individuals with which the outbred individual interacts. If so, such effects of inbreeding may be considered a form of indirect genetic effects, which occur when a trait expressed in a focal individual is influenced by genes in another individual with which the focal individual interacts (Moore *et al.*, 1997). However, it should be noted that, in the case of inbreeding, these indirect genetic effects are based on dominance effects rather than on additive genetic effects, as is the case for classical indirect genetic effects. Thus, we encourage further work on inbreeding to examine indirect effects in a wide range of social contexts, including competition, social dominance and aggressive interactions, and communal and cooperative breeding.

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